U.PORTO

DISSERTAÇÃO DE MESTRADO

TOXICOLOGIA E CONTAMINAÇÃO AMBIENTAIS

Marine Actinobacteria from the Northern Portuguese Coast: isolation, identification and investigation of their bioactive potential

Inês Filipa Coelho Ribeiro

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2017

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M.ICBAS 2017

SEDE ADMINISTRATIVA

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

FACULDADE DE CIÊNCIAS

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MARINE ACTINOBACTERIA FROM THE NORTHERN PORTUGUESE COAST: ISOLATION, IDENTIFICATION AND INVESTIGATION OF THEIR BIOACTIVE POTENTIAL

Dissertação de Candidatura ao grau de Mestre em Toxicologia e Contaminação Ambientais submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor, Dr. Fátima Carvalho, who received me and made possible the work done in this thesis. My genuine thanks for all the shared knowledge, for all the trust and dedication and for all you provided me so that my goals were achieved. Thank you for being part of a very important phase for my personal and professional development.

I would also like to thank my co-advisor, Dr. Filipe Pereira for his trust, for the support he provided me and for his contribution in the tools of molecular biology that were used in this work.

Then I would like to thank Dr. Ana Paula Mucha for the opportunity to join the EcoBioTec Laboratory, where I have always been well treated and the best conditions necessary for the accomplishment of my work have been provided. I would also like to thank CIIMAR - Interdisciplinary Centre of Marine and Environmental Research for the equipment and installations.

My colleagues at the Ecobiotec laboratory, especially Filipa Santos, Joana Fernandes, Rafaela Mendes, Diogo Alexandrino, Adriana Rego and Teresa Martins, thank you for being always present and supporting me in the most difficult situations, sharing knowledge and above all for your precious friendship.

I am also grateful to Dr. Pedro Leão and Dr. Ralph Urbatzka for their help in the screening of antimicrobial and anticancer activities.

To my family I owe everything I am today, especially to my parents and sister. I have no words to thank you for all the strength, patience and understanding at this point in my life. A big thank you for always helping me to achieve my dreams. Finally, I would like to thank my boyfriend, António Rodrigues, for all the patience, dedication and affection, for all the words of support and for being present in my life.

I would also like to acknowledge the financial support of ICBAS for attending the 7th Congress of European Microbiologists – FEMS and of project INNOVMAR - Innovation and Sustainability in the Management and Exploitation of Marine Resources (reference NORTE-01-0145-FEDER-000035, within Research Line NOVELMAR), supported by North Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

ABSTRACT

There is a great need to find treatments that are more effective and less aggressive against serious diseases, such as multidrug-resistant bacterial infections and cancer. Natural compounds are being increasingly used in biotechnological applications and a large fraction of these substances is channeled to the pharmaceutical industry due to their important bioactive properties. The discovery of new bioactive compounds with novel mechanisms of action constitutes a promising solution for the designing of new therapeutic solutions.

Actinobacteria are a group of Gram-positive and filamentous bacteria well known for their unmatched capacity to produce a vast range of bioactive compounds with high industrial and pharmaceutical importance. In recent years a great interest has emerged in the research of marine actinobacteria for the discovery of new bioactive molecules, mainly as a consequence of the overexploitation of this group of bacteria in terrestrial environments. The Portuguese coast is scantly explored in terms of actinobacterial diversity and of their bioactive potential. Such unexplored environments offer a great opportunity to obtain new marine actinobacteria, also increasing the chances of finding new natural products with bioactivity.

The present master's thesis aimed at the isolation of actinobacteria from marine environments of the Portuguese northern coast and investigation of their potential to produce bioactive compounds with antimicrobial or anticancer activities.

The first study of this thesis consisted in the investigation of actinobacteria associated with a sediment sample collected from the intertidal zone of a beach located on Parque Natural do Litoral Norte, through a cultivation-dependent approach, and in the screening of the bioactive potential of the obtained actinobacterial isolates. To improve the efficiency of actinobacteria isolation, three different treatment methods and three selective culture media were used. A total of 117 isolates were obtained, of which 52 strains were assigned to the phylum Actinobacteria. Seven different genera of actinobacteria could be identified, with the largest fraction of the isolates belonging to the genus *Micromonospora*. Some non-actinobacterial isolates were also obtained, belonging to the phyla Firmicutes, Proteobacteria and Bacteroidetes. Crude extracts of all actinobacterial isolates were screened for antimicrobial and anticancer activities. Some strains identified as *Streptomyces* and *Micromonospora* species showed promising antimicrobial and

anticancer activities. The results obtained demonstrate the importance of bioprospecting other coastal marine sediments in Portugal.

The second study presented in this thesis focused on the isolation and identification of rare actinobacteria from the marine sponge *Hymeniacidon perlevis*, collected on the intertidal zone of a beach on the northern Portuguese coast. To increase the isolation success of rare actinobacteria, three selective pretreatments and culture media were employed. In total, 188 strains were isolated but only 18 were possible to be identified to date. Identification results showed that the experimental approach used allowed the isolation of rare genera of actinobacteria such as *Nocardia*, *Nocardiopsis*, *Brevibacterium*, *Gordonia* and *Tsukamurella*, which are very promising concerning the production of bioactive compounds. The results so far obtained also suggest that the studied sponge appears to be rich in actinobacteria.

In the future, it will be necessary to better investigate the antimicrobial and anticancer activities of the crude extracts obtained in the first study and find if these extracts may contain new bioactive molecules. It will be also important to identify the remaining isolates derived from the second study and screen for their ability to produce bioactive compounds

In general, the results obtained in this thesis are a good contribution to the scarce information available in the literature on actinobacteria associated with marine sediments and sponges of the northern coast of the country, as well on their bioactive potential.

RESUMO

Existe uma grande necessidade de encontrar tratamentos mais eficazes e menos agressivos contra doenças graves, como infeções provocadas por bactérias resistentes a múltiplos fármacos e cancro. Os compostos naturais são cada vez mais utilizados em aplicações biotecnológicas, sendo uma grande fração dessas substâncias canalizada para a indústria farmacêutica devido às suas importantes propriedades bioativas. A descoberta de novos compostos bioativos com novos mecanismos de ação constitui uma solução promissora para a conceção de novas soluções terapêuticas.

As actinobactérias são um grupo de bactérias Gram-positivas e filamentosas bem conhecidas pela sua capacidade inigualável de produção de uma vasta gama de compostos bioativos, com elevado interesse industrial e farmacêutico. Nos últimos anos, foi despoletado um grande interesse pela investigação de actinobactérias marinhas para a pesquisa de novas moléculas bioativas, principalmente devido à sobre exploração deste grupo de bactérias em ambientes terrestres. A costa portuguesa encontra-se muito pouco explorada em termos da sua diversidade actinobacteriana e do seu potencial para produzir compostos bioativos. Tais ambientes inexplorados oferecem uma ótima oportunidade para obter novas actinobactérias marinhas, aumentando também as chances de se encontrar novos produtos naturais com bioatividade.

A presente tese de mestrado tem como objetivo o isolamento de actinobactérias de ambientes marinhos da costa norte Portuguesa e investigação do seu potencial para produzir compostos bioativos com atividades antimicrobianas ou anticancerígenas.

O primeiro estudo desta tese consistiu na investigação de actinobactérias associadas a uma amostra de sedimento recolhida na zona intertidal de uma praia localizada no Parque Natural do Litoral Norte, através de uma abordagem dependente de cultivo, e no estudo do potencial bioativo dos isolados de actinobactérias obtidos. De forma a aumentar a eficácia do isolamento de actinobactérias, foram utilizados três métodos de tratamento diferentes e três meios de cultura seletivos. No total, 117 isolados foram obtidos, dos quais 52 estirpes se verificaram pertencerem ao filo Actinobacteria. Foram identificados sete géneros diferentes de actinobactérias, com a maior fração dos isolados pertencendo ao género *Micromonospora*. Foram também obtidos alguns isolados não-actinobacterianos, pertencentes aos filos Firmicutes, Proteobacteria e Bacteroidetes. Os extratos brutos de todos os isolados actinobacterianos foram analisados quanto às suas atividades antimicrobianas e anticancerígenas. Algumas estirpes identificadas como

espécies de *Streptomyces* e *Micromonospora* mostraram atividades antimicrobianas e anticancerígenas promissoras. Os resultados obtidos demonstram a importância da bioprospecção de outros sedimentos marinhos costeiros em Portugal.

O segundo estudo apresentado nesta tese focou-se no isolamento e identificação de actinobactérias raras da esponja marinha *Hymeniacidon perlevis*, recolhida na zona intertidal de uma praia na costa norte do país. Para aumentar o sucesso de isolamento de actinobactérias raras, foram utilizados três pré-tratamentos e meios de cultura seletivos. No total, 188 estirpes foram isoladas, mas apenas 18 foram possíveis de ser identificadas até a data. Os resultados de identificação mostraram que a abordagem experimental utilizada permitiu o isolamento de géneros raros de actinobactérias como *Nocardia*, *Nocardiopsis*, *Brevibacterium*, *Gordonia* e *Tsukamurella*, os quais são muito promissores quanto à produção de compostos bioativos. Os resultados obtidos até agora também sugerem que a esponja estudada parece ser rica em actinobactérias.

No futuro, será necessário investigar melhor as atividades antimicrobianas e anticancerígenas dos extratos brutos obtidos no primeiro estudo e verificar se esses extratos podem conter novas moléculas bioativas. Também será importante identificar os restantes isolados derivados do segundo estudo e estudar a sua capacidade de produzir compostos bioativos.

Em geral, os resultados obtidos nesta tese são um bom contributo para a escassa informação disponível na literatura sobre actinobactérias associadas a sedimentos marinhos e esponjas da costa norte do país, bem como sobre o seu potencial bioativo.

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CHAPTER 1

INTRODUCTION

1. Introduction

The search for new drugs to combat diseases for which treatments are inexistent or no longer effective is an urgent topic. Cancer and multidrug-resistant bacterial infections are at the top of diseases for which new therapies are needed. As health problems represent a constant threat to humanity, it is necessary to continuously seek for new solutions, one of which is the discovery of new bioactive compounds (Duncan et al., 2015).

Actinobacteria are an extremely important source of diverse bioactive compounds with industrial and pharmaceutical interest (Claverias et al., 2015; Stach et al., 2003). For more than 50 years, actinobacteria have been studied due to their unique ability to produce bioactive metabolites relevant for human health, such as: antibiotics (e.g., amphotericin B, erythromycin), antifungal agents (e.g., chandrananimycin A), antiparasitic agents (e.g., avermectins), antiviral agents (e.g., benzatatin C), anticancer agents (e.g., daunorubicin, bleomycin, mitomycin), immunosuppressive agents (e.g., papamycin) and anti-inflammatory agents (e.g., cyclomarin A, salinamides A and B). They are also important producers of enzymes (e.g., amylase, protease, cellulase, halogenases), enzyme inhibitors (e.g., pyrizinostatin, pyrostatin), fungicides (e.g., nystatin), pigments (e.g., dark pigments, melanin or melanocytes), herbicides (e.g., herbicide A and B, anisomycin) and plant and animal growth promoting substances (Donadio et al., 2002; Manivasagan et al., 2014; Pimentel-Elardo et al., 2010; Subramani & Aalbersberg, 2012).

Production of bioactive compounds has been mostly explored in terrestrial actinobacteria, perhaps due to the easier access to samples, however the constant search of terrestrial environments has often led to the re-isolation of known phylotypes, making it difficult to find novel species and, consequently, new bioactive compounds (Zotchev, 2012).

Compared to terrestrial environments, oceans support a much greater biodiversity and cover more than 70% of the terrestrial surface (Donia & Hamann, 2003). Due to the profoundly different characteristics of the terrestrial environments compared with marine ecosystems, it is reasonable to expect that oceans harbour distinct marine actinobacteria with the capacity to produce novel bioactive compounds (Manivasagan et al., 2013). Marine actinobacteria are widely distributed in the oceans and can be found in fish, sponges, seaweed, mollusks, seawater, sediments, etc. In the recent years, these microorganisms have incited the interest of researchers, not only for their taxonomic diversity and ecological importance but also for the possibility of biosynthesis of new natural products (Dharmaraj, 2010). However, despite a more intense research on marine

actinobacteria and isolation efforts, the oceans still remain underexplored ecosystems with respect to the diversity of these microorganisms (Stach & Bull, 2005) and of their metabolites produced (Manivasagan et al., 2014).

1.1. Phylum Actinobacteria

The phylum Actinobacteria constitutes one of the largest taxonomic units, either in number and diversity, within the main known lineages that integrate the domain Bacteria (Ventura et al., 2007). It covers a vast group of Gram-positive bacteria with diverse morphological and physiological characteristics, usually sharing a high genomic guanine+citosine content, which can vary from 50% to more than 70% (Barkal et al., 2016; Ventura et al., 2007).

The taxonomy of this phylum has been recently reviewed and, according to this update, the phylum now includes the classes *Actinobacteria*, *Acidimicrobidiia*, *Coriobacteriia*, *Nitriliruptoria*, *Rubrobacteria*, and *Thermoleophilia* (Ludwig et al., 2015). The class *Actinobacteria* integrates most of the taxonomic units responsible for the production of bioactive compounds, commonly known as actinomycetes, and it comprehends 15 orders: *Actinomycetales*, *Actinopolysporales*, *Bifidobacteriales*, *Catenulisporales*, *Corynebacteriales*, *Frankiales*, *Glycomycetales*, *Jiangellales*, *Kineosporiales*, *Micrococcales*, *Micromonosporales*, *Propionibacteriales*, *Pseudonocardiales* *Streptomycetales* and *Streptosporangiales.* In this thesis, the term actinobacteria will be used as synonymous of the class *Actinobacteria.*

The diversity of species of actinobacteria includes pathogens (e.g., *Corynebacterim*, *Mycobacterium*, *Nocardia*, *Propionibacterium* and *Thropherma* spp.), soil or aquatic inhabitants (e.g., *Micromonospora, Rhodococcus, Salinispora* and *Streptomyces* spp.), plant pathogens (e.g., *Leifsonia* spp.), nitrogen-fixing plant symbionts (*Frankia* spp.) and inhabitants of the gastrointestinal tract (e.g., *Bifidobacterium* spp.) (Barka et al., 2016; Ventura et al., 2007). Actinobacteria present a great diversity of morphologies, such as coccoid (*Micrococcus* spp.) or rod-coccoid (e.g., *Arthrobacter* spp.), and also contemplate species where hyphae fragmentation occurs (e.g., *Nocardia* spp.) or that are differentiated into branched mycelium, similar to fungal hyphae (e.g., *Streptomyces* spp.) (Barka et al., 2016; Ventura et al., 2007). Some species have the capacity to reproduce through the production of spores, sporangiospores or conidiophores. Spores are the main form of

reproduction and are produced on a large scale. Each spore has the possibility of germinating and growing so that a new microorganism is developed. Although not very resistant to heat, sporangiospores and conidiophores are resistant to dehydration and aid in the survival of species during drought. Other species, such as the genus *Nocardia*, multiply by fragmentation of hyphae in various bacilliform cells or cocci, each with the capacity to produce a new mycelium (Ventura et al., 2007).

Actinobacteria are mostly aerobic, although some genera may be microaerophilic or anaerobic (Ventura et al., 2007). Metabolically, they can be heterotrophic, autotrophic, chemotrophic or phototrophic (Goodfellow & Williams, 1983; Stach & Bull, 2005). In addition, these microorganisms exhibit important metabolic traits, like the production of molecules with relevant bioactive properties, extracellular enzymes and various types of pigments (Schrempf, 2001).

1.2. Environmental distribution of actinobacteria

Actinobacteria have adapted to many ecosystems and are present in both terrestrial and aquatic (fresh and salt water) environments and in the air. However, they are more predominant in the soil, where they have an important contribution for organic matter decomposition, including environmental pollutants (Ventura et al., 2007). This group of microorganisms was originally isolated from soils and was proven to be very important for human and veterinary medicine, biotechnology and ecology, mainly because these microorganisms are a source of compounds with high bioactive potential (Barka et al., 2016). More than 10 000 of currently known antibiotics are derived from the secondary metabolism of several species of actinobacteria (Montalvo et al., 2005). Due to the remarkable history of actinobacteria as producers of bioactive compounds, considerable efforts have been made by the scientific community to isolate new actinobacteria for drug screening programs (Montalvo et al., 2005).

Until recently, actinobacteria were only considered soil-dwelling microorganisms. The isolation of these microorganisms from marine samples initially led to the idea that they would come from resistant spores of terrestrial actinobacteria. However, Jensen et al. (1991) provided scientific evidence that actinobacteria isolated from tropical marine sediments were native to the marine environment, thus making this ecosystem an attractive and promising source of new actinobacteria.

1.3. Marine environment

The marine environment can be separated into coastal habitats and open ocean habitats. These habitats can range from the sea surface to the pelagic zone, which begins below the tidal influence zone, extending to the high seas, reaching depths of over 10 000 meters (Ward & Bora, 2006). The seafloor is constituted by sediments of diverse geology, carbonate structures, mineral nodules fields, cold seeps, saturated brines, hydrocarbon infiltrations and hydrothermal vents. It presents a micro and macrofauna with diverse biotic relationships, such as symbiosis and epibiosis (Ward & Bora, 2006). Oceans are ecosystems with unique characteristics in terms of pressure, salinity, temperature and oxygen concentrations (Manivasagan et al., 2013). These environmental pressures together with the huge biodiversity found in these ecosystems, have fostered the development over the years of distinct microbial physiological characteristics to ensure survival of microorganisms in this dynamic environment, one of them being the production of a high diversity of bioactive metabolites (Gulder & Moore, 2009).

Due to its distinctive characteristics, the marine environment is therefore an excellent source to explore new species of actinobacteria (Manivasagan et al., 2014). These microorganisms are very important components of marine bacterial communities, presenting a wide distribution throughout marine ecosystems (Fig.1) (Abdelmohsen et al., 2014). In recent years, new species of marine actinobacteria with unique metabolic capabilities have been discovered (Fenical & Jensen, 2006). Therefore, the proven indigenous character of marine actinobacteria, their high abundance and diversity, together with their prodigious capacity to produce secondary metabolites, make the marine environment a wealthy source of new bioactive compounds (Das et al., 2007; Jensen et al., 2005; Subramani & Aalbersberg, 2012).

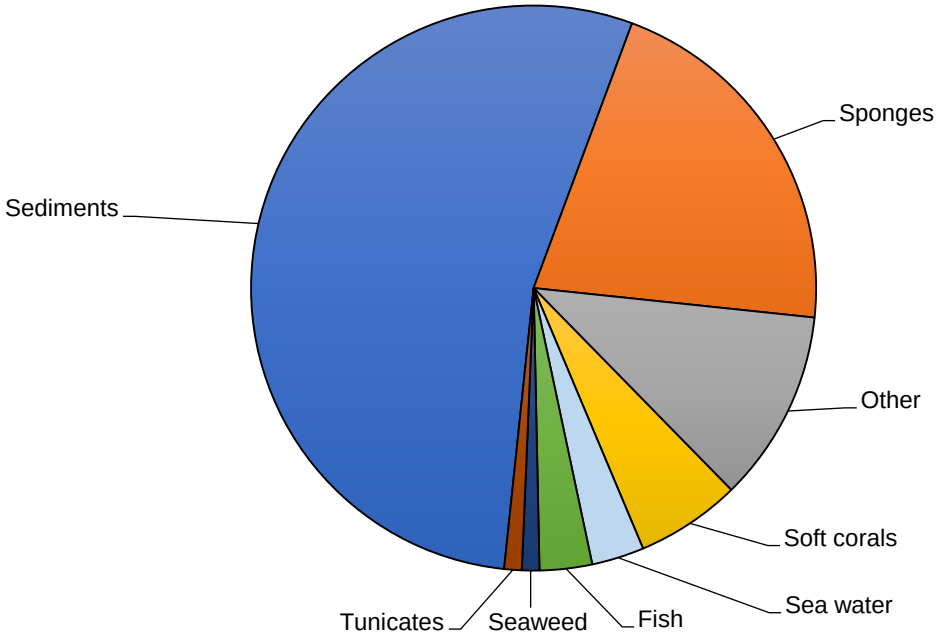

Figure 1 - Distribution of actinobacteria in the marine environment (the study took into account a total of 10.400 16S rRNA gene sequences of marine actinobacteria). Adapted from Abdelmohsen et al. (2014).

1.4. Marine sediments

Marine sediments represent one of the major biomes on Earth, however little is known about their taxonomic and functional microbial diversity. They have a number of microbial cells many times higher than seawater, being considered one of the marine habitats with the highest rates of microbial diversity. The characteristics of marine sediments promote the development of microenvironments where bacteria interact and compete trough mechanisms such as the production of secondary metabolites (Patin et al., 2017).

The research of actinobacteria in marine sediments has been a subject of attention in the last decade due to a significant development of techniques for the isolation and identification of native marine microflora. As a result, novel genera of actinobacteria have emerged, having a great potential for the discovery of new bioactive compounds with pharmaceutical and industrial interest (Jensen et al., 2005). Examples of these promising

novel genera include: *Salinispora*, *Demequina, Aestuariimicrobium*, *Sciscionella, Marinactinospora*, *Paraoerskovia*, *Marisediminicola*, *Miniimonas*, *Spinactinospora*, *Sediminihabitans*, *Flaviflexus*, *Mariniluteicoccus* and *Halopolyspora* (Claverias et al., 2015). Knowledge of actinobacterial diversity in marine sediments has increased thanks to the cultivation of these microorganisms from marine sediment samples taken from various depths (Maldonado et al., 2005b). Thus, it has been possible to improve the knowledge on the taxonomy and geographical distribution of these microorganisms as well as to isolate new actinobacteria with biotechnological potential (Maldonado et al., 2005a; Maldonado et al., 2005b; Ward & Bora, 2006)

1.5. Sponges

The phylum Porifera, commonly known as sponges, includes the oldest animal group living on our planet. Sponges mainly inhabit marine habitats, but may also be present in fresh waters. They are sedentary feeders, with most marine sponges being benthonic and filtering organisms. Capable of pumping thousands of liters of water per day, sponges capture planktonic organisms from seawater through continuous water transportation to their interior (Hentschel et al., 2012; Van Soest et al., 2012).

Over the years, sponges have established great symbiotic relationships with microorganisms (bacteria, archaea and fungi). The microbial biomass can constitute up to 35% - 40% of the sponge's volume (Schmitt et al., 2012; Webster & Taylor, 2012) . So far, 32 bacterial phyla have been reported to be associated with marine sponges, with the most *abundant* being *Proteobacteria, Actinobacteria, Chloroflexi, Firmicutes, Bacteriodetes, Cyanobacteria and Acidobacteria* (Fig.2) (Schmitt et al., 2012).

Marine sponges have a well-developed immune system. For their defense, marine sponges produce a wide range of bioactive compounds to protect against various threats, such as predators, microbial infestations, to prevent the growth of sessile organisms etc. (Hentschel et al., 2012; Suthindhiran et al., 2014), though scientific evidences indicate that many of these compounds may be actually produced by their symbiont microorganisms (Mehbub et al., 2014). Together, marine sponges and their symbiotic microorganisms have a remarkable chemical repertoire, which has been drawing the attention of the pharmaceutical industry (Hentschel et al., 2012).

Marine actinobacteria have been isolated from diverse species of sponges and these organisms have been found to host important actinobacterial genera, as is the case of the novel genus *Salinospora* (Kim et al., 2006). In addition, sponge-associated actinobacteria have been reported to produce secondary metabolites with a wide range of biological activities (Abdelmohsen et al., 2014).

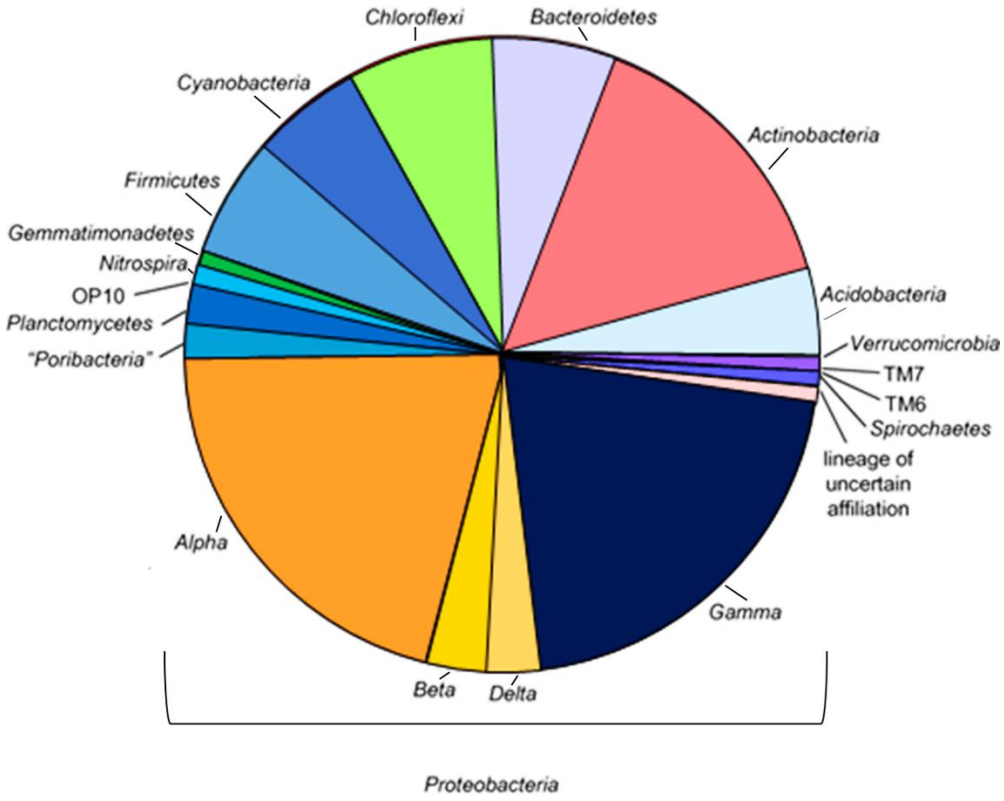

Figure 2 - Phylogenetic distribution of sponge-associated bacteria (considering a total of 11 284 16S rRNA gene sequences retrieved from GenBank). Source: (Webster & Taylor, 2012).

1.6. Marine actinobacteria

Marine actinobacteria are known for their great ability to produce secondary metabolites with biological activity. The potential is so high that a single strain may have the ability to produce 10-20 different secondary metabolites. It is estimated that about 25 000 – 27 000 antibiotics are produced by microorganisms and that from these, about 10 000 (ca. 40%) are produced by actinobacteria (Berdy, 2012). Approximately 67% of the natural products produced by marine actinobacteria come from microorganisms isolated from Coral Sea,

Yellow Sea, Indian Ocean, Mediterranean Sea and Pacific Ocean (Manivasagan et al., 2013).

Several studies have shown the existence of actinobacteria native to the marine environment, belonging to various genera, such as, *Micromonospora*, *Streptomyces*, *Actinomadura, Actinosynnema, Amycolatopsis, Kitasatospora, Micrococcus, Microbacterium, Nocardioides, Nocardiopsis, Nonomurea, Tsukamurella,* and *Williamsia* (Manivasagan et al., 2014; Ward & Bora, 2006).

Recently the genera *Salinispora*, *Marinispora* and *Verrucosispora* have also been reported, and their members were found to require sea water to grow. The genus *Salinibacterium* is another example recently reported, whose species tolerate NaCl concentrations up to 10%, but do not require the presence of salt for their growth (Bruns et al., 2003; Han et al., 2003; Kwon et al., 2006a; Maldonado et al., 2005a; Riedlinger et al., 2004). Many of these genera are capable of producing unique bioactive compounds such as antimicrobial, antifungal, anticancer, antitumor, anti-inflammatory, anti-viral, anti-malarial, antioxidant, cytotoxic, antialgal and anti-angiogenesis agents (Table 1) (Manivasagan et al., 2014).

Table 1. Examples of secondary metabolites produced by marine actinobacteria *(*adapted from Manivasagan et al. 2014). Different colors in the Table indicate different bioactivities

Compound	Species	Biological activity	Reference
Abyssomicins	*Verrucosispora* sp.	Antibacterial	Riedlinger et al. (2004)
Bonactin	*Streptomyces* sp.	Antibacterial; antifungal	Schumacher et al. (2003)
Chloro-dihydroquinones	*Streptomyces* sp.	Antibacterial; anticancer	Soria-Mercado et al. (2005)
Diazepinomicin	*Micromonospora* sp.	Antibacterial; anticancer; Anti-inflammatory	Charan et al. (2004)
Frigocyclinone	*Streptomyces griseus*	Antibacterial	Bruntner et al. (2005)
Essramycin	*Streptomyces* sp.	Antibacterial	El-Gendy et al. (2008)
Lynamicins	*Marinispora* sp.	Antibacterial	McArthur et al. (2008)
Marinopyrroles	*Streptomyces* sp.	Antibacterial; cytotoxic	Hughes et al. (2008)
Caboxamycin	*Streptomyces* sp.	Antibacterial; cytotoxic	Hohmann et al. (2009c)
Himalomycins	*Streptomyces* sp.	Antibacterial	Maskey et al. (2003a)

Marinomycins	*Marinispora*	Antibacterial; antifungal; anticancer	Kwon et al. (2006b)
Glyciapyrroles	*Streptomyces* sp.	Antibacterial	Macherla et al. (2005)
Tirandamycin	*Streptomyces* sp.	Antibacterial	Carlson et al. (2009)
Bisanthraquinone	*Streptomyces* sp.	Antibacterial	Socha et al. (2006)
Gutingimycin	*Streptomyces* sp.	Antibacterial	Maskey et al. (2004b)
Helquinoline	*Janibacter limosus*	Antibacterial	Asolkar et al. (2004)
Lajollamycin	*Streptomyces nodosus*	Antibacterial	Manam et al. (2005)
TP-1161	*Nocardiopsis* sp.	Antibacterial	Engelhardt et al. (2010)
Lincomycin	*Streptomyces lincolnensis*	Antibacterial	Peschke et al. (1995)
1,4-Dihydroxy-2-(3-hydroxybutyl)-9,10-anthraquinone 9,10-anthrac	*Streptomyces* sp.	Antibacterial	Ravikumar et al. (2012b)
Chandrananimycin	*Actinomadura sp*	Antifungal; antialgal; antibacterial; anticancer	Maskey et al. (2003b)
N-(2-hydroxyphenyl)-2-phenazinamine (NHP)	*Nocardia dassonvillei.*	Antifungal; anticancer	Gao et al. (2012)
Salinosporamide A	*Salinispora tropica*	Anticancer	Feling et al. (2003)
Caprolactones	*Streptomyces* sp.	Anticancer	Stritzke et al. (2004)
3,6-Disubstituted indoles	*Streptomyces* sp.	Anticancer	López et al. (2003)
IB-00208	*Actinomadura* sp.	Anticancer	Rodriguez et al. (2003)
1- Hydroxy-1-norresistomycin	*Streptomyces chinaensis*	Anticancer; antibacterial	Gorajana et al. (2005) and Kock et al. (2005)
ZHD-0501	*Actinomadura* sp.	Anticancer	Han et al. (2005)
Chinikomycins	*Streptomyces* sp.	Antitumor	Li et al. (2005)
Glyciapyrroles	*Streptomyces* sp.	Antitumor	Macherla et al. (2005)
Mechercharmycin A	*Thermoactinomyces sp*	Antitumor	Kanoh et al. (2005)
Aureoverticillactam	*Streptomyces aureoverticillatus*	Antitumor	Mitchell et al. (2004)
Arenicolides	*Salinispora arenicola*	Antitumor	Williams et al. (2007)
Chalcomycin	*Streptomyces* sp.	Antitumor	Asolkar et al. (2002)
Daryamides	*Streptomyces* sp.	Antitumor	Asolkar et al. (2006)
Aureolic acid	*Streptomyces* sp.	Antitumor	Lu et al. (2012)
1,8-Dihydroxy-2-ethyl-3-methylanthraquinone	*Streptomyces* sp.	Antitumor	Huang et al. (2006)

Arenimycin	*Salinispora arenicola*	Antitumor; antibacterial	Asolkar et al. (2010b)
Mitomycin C	*Streptomyces lavendulae*	Antitumor	Mao et al. (1999)
Staurosporinone	*Streptomyces* sp.	Antitumor; phycotoxicity	Wu et al. (2006)
Streptokordin	*Streptomyces* sp.	Antitumor	Jeong et al. (2006)
Elaiomycins B and C	*Streptomyces* sp.	Antitumor	Helaly et al. (2011)
Manumycins	*Streptomyces* sp.	Cytotoxic	Chauhan et al. (2005)
Salinipyrones	*Salinispora pacifica*	Cytotoxic	Oh et al. (2008)
Pacificanones	*Salinispora pacifica*	Cytotoxic	Oh et al. (2008)
Actinofuranones	*Streptomyces*	Cytotoxic	Cho et al. (2006)
Nonactin	*Streptomyces* sp.	Cytotoxic	Jeong et al. (2006)
Resistoflavine	*Streptomyces chibaensis*	Cytotoxic	Gorajana et al. (2007)
Neomarinones	*Actinomycetales*	Cytotoxic	Hardt et al. (2000)
Piericidins	*Streptomyces* sp.	Cytotoxic	Hayakawa et al. (2007)
Lucentamycins	*Nocardiopsis lucentensis*	Cytotoxic	Cho et al. (2007)
Arenamides	*Salinipora arenicola*	Cytotoxic	Asolkar et al. (2010a)
Piperazimycins	*Streptomyces* sp.	Cytotoxic	Miller et al. (2007)
Mansouramycin C	*Streptomyces* sp.	Cytotoxic	Hawas et al. (2009)
Usabamycins	*Streptomyces* sp.	Cytotoxic	Sato et al. (2011)
Pyridinium	*Amycolatopsis alba.*	Cytotoxic; antimicrobial	Dasari et al. (2012)
ML-449 (macrolactam)	*Streptomyces* sp.	Cytotoxic	Jørgensen et al. (2010)
Salinosporamide B & C	*Salinipora tropica*	Cytotoxic	Williams et al. (2005)
Albidopyrone	*Streptomyces* sp.	Cytotoxic (inhibitor of protein–tyrosine phosphatase)	Hohmann et al. (2009a)
Proximicins	*Verrucosispora* sp.	Cytostatic	Schneider et al. (2008)
Cyclomarins	*Streptomyces* sp. *and Salinispora Arenicola*	Anti-inflammatory	Renner et al. (1999) and Schultz et al. (2008)
Salinamides A and B	*Streptomyces* sp.	Anti-inflammatory	Moore et al. (1999)
Avermectins	*Streptomyces avermitilis*	Anti-inflammatory	Burg et al. (1979)
Trioxacarcin	*Streptomyces* sp.	Anti-malarial; antibacterial; antitumor	Maskey et al. (2004a)

Benzastatin C	*Streptomyces nitrosporeus*	Anti-viral	Lee et al. (2007)
Dermacozines A-G	*Dermacoccus*	Antioxidant; antitumor;	Abdel-Mageed et al. (2010)
Lipocarbazoles	*Tsukamurella pseudospumae*	Antioxidant	Schneider et al. (2009)
2- Allyloxyphenol	*Streptomyces* sp.	Antioxidant; antimicrobial	Arumugam et al. (2010)
Streptopyrrolidine	*Streptomyces* sp.	Anti-angiogenesis	Shin et al. (2008)
Cyclo-(l-Pro-l-Met)	*Nocardiopsis* sp.	Anti-angiogenesis	Shin et al. (2010)

1.7. Marine rare actinobacteria

Due to the fact that, among actinobacteria, *Streptomyces* species are the most frequently isolated strains, isolation of other actinobacterial genera is an important step towards the discovery of new bioactive compounds (Cai et al., 2009). Rare actinobacteria are generally known as non-*Streptomyces* species, which frequency of isolation using traditional culturing methods is much lower comparing with *Streptomyces* strains. These microorganisms are reported to be well distributed in both terrestrial and aquatic ecosystems (Tiwari & Gupta, 2012).

The research of rare actinobacteria combined with the exploration of poorly studied natural ecosystems has resulted in the isolation of approximately 220 rare genera, with a large fraction of these being responsible for the production of about 3000 bioactive compounds (Berdy, 2012; Tiwari & Gupta, 2012). In the marine environment, rare actinobacteria have been isolated from sediments, sea water, sponges, tunicates, seaweeds and marine mollusks, but a vast majority of marine ecosystems remain unexplored in terms of these bacteria (Dhakal et al., 2017; Subramani & Aalbersberg, 2013). Examples of rare marine actinobacteria genera include *Nocardia*, *Nocardiopsis*, *Actinomadura*, *Micromonospora*, *Marinactinospora, Salinispora, Actinoalloteichus* and *Saccharomonospora,* these microorganisms are responsible for the production of important bioactive compounds such as peptidolipins, with antibacterial activity against multiresistant bacteria, isomethoxyneihumicin, which has high cytotoxicity against leukemia cells, halomadurone, used for the treatment of neurodegenerative diseases, anthracyclinones, with cytotoxicity against the human colon adenocarcinoma cell line, marthiapeptide A show antibacterial activity against Gram-positive bacteria and high cytotoxic activity, salinoquinones with

cytotoxic activity, caerulomycins exhibit cytotoxic activity against diverse cancerous cell lines and taromycin which show antibacterial activity (Dhakal et al., 2017; Fu et al., 2011; Fukuda et al., 2017; Liang et al., 2016; Murphy et al., 2010; Sousa et al., 2012; Wyche et al., 2012; Wyche et al., 2013; Yamanaka et al., 2014; Zhou et al., 2012).

1.8. Ecologic importance of actinobacteria in the marine environment

The degradation and transformation of organic matter is a continuous process carried out by several microorganisms (Bruns et al., 2003; Heald et al., 2001). In addition to the high capacity for producing bioactive compounds, actinobacteria also play a key role in the recycling of nutrients in marine ecosystems, being essential for the mineralization of organic matter and fixation of mineral nutrients and nitrogen (Das et al. 2007; Haefner, 2003).

Several marine actinobacteria capable of degrading compounds such as agar, alginate, laminarin, cellulose, chitin (Pisano et al., 1992), petroleum and other hydrocarbons have been described (Manivasagan et al., 2013; Pisano et al., 1992). The genera *Arthobacter*, *Brevibacterium*, *Corynebacterium* and *Nocardia* are considered important microorganisms in the degradation of petroleum (Goodfellow & Williams, 1983). In addition, marine actinobacteria have also been reported to be capable of producing enzymes of great industrial and pharmaceutical interest (Ramesh & Mathivanan, 2009). Thus, it is important to assess the ecological role of marine actinobacteria, not only because of their ability to produce secondary metabolites with high biotechnological potential, but also due to their biodegradation capacity which can be exploited for the bioremediation of various pollutants.

1.9. Production of secondary metabolites by actinobacteria

Actinobacteria produce a diverse array of secondary metabolites with high biotechnological relevance and their production may be increased through the optimization of fermentation conditions (Sujatha et al., 2005). Synthesis of secondary metabolites is only initiated when growth of these microorganisms starts to slow down or is stopped (after primary metabolism). For this reason, the synthesis of these metabolites is influenced by factors directly related to microbial growth, such as nutrient availability, nitrogen or

phosphorus concentration in the medium, pH, temperature, oxygen partial pressure, agitation, mineral salts and metal ions available and presence of precursors, inducers or inhibitors, (Manivasagan et al., 2013). The synthesis of secondary metabolites by actinobacteria is related to cell growth as follows: (i) adaptation phase (lag phase) - growth is almost absent and protein production is induced as a response to surrounding conditions; (ii) exponential growth phase (log phase) - there is an intense microbial growth, with synthesis of secondary metabolites being scarce; (iii) transition phase - growth and protein synthesis are repressed, synthesis of secondary metabolism enzymes is intensified thus initiating the production of secondary metabolites; (iv) production phase - growth rate is reduced, dry weight remains similar and the production of secondary metabolites is maximal (Bibb, 2005; Demain, 1998; Manivasagan et al., 2013).

The production of these secondary metabolites is mediated by large enzymatic complexes, namely polyketide synthases (PKS) and nonribosomal peptide synthases (NRPS). PKS mediate the production of polyketides through sequential decarboxylative condensations of small building blocks of acetate or other small carboxylic acids. An individual PKS enzyme may consist in one or more functional modules, which comprise several domains, such as β-ketoacyl synthase, acyltrasnferase and acyl carrier proteins. Most of these enzymes also contain optional domains, such as β-ketoacyl reductase, dehydratase, enoylreductase and methyltransferase (Du & Lou, 2010; Khan et al., 2014). NRPS are a group of multifunctional enzymes, which synthesize peptides in a modular structure, through several domains such as adenylation, condensation and peptidyl carrier proteins, though other domains may be also associated with these enzymes (Du & Lou, 2010; Jenke-Kodama & Dittmann, 2009).

The molecules built by PKS or NRPS are then finished by tailoring enzymes, such as glycosyltransferases, methyltransferases, acyltransferases, prenyltransferases, transaminases, cyclases, halogenases, ketoredutases and oxygenases. Within these reactions, halogenation is a particularly important reaction for the bioactivity of a large number of natural compounds (Li et al., 2013).

1.9.1. Secondary metabolites with antibacterial activity

Bacterial infections by antibiotic-resistant bacteria are one of the leading causes of death worldwide. In order to overcome this problem, it is urgent to discover new effective

antibacterial substances with minimal side effects (Manivasagan et al., 2014; Ravikumar et al., 2012a)

Marine actinobacteria have been found to be a potent resource of secondary metabolites with antibacterial activity. Examples include Abyssomycin C, a polycyclic polyethylene antibiotic produced by a marine strain of *Verrucosispora* sp. (Riedlinger et al., 2004), acting against Gram-positive clinical isolates (Hassan et al., 2017; Manivasagan et al., 2014); Bonactin, isolated from the liquid culture of a *Streptomyces* sp. strain, exhibiting antimicrobial activity against Gram-negative bacteria and fungi; Diazepinomycin produced by a *Micromonospora* sp., with antibacterial activity against Gram-positive bacteria (Charan et al., 2004); Essramycin, an antibacterial compound isolated from a strain of *Streptomyces griseus* and with a high activity against Gram-positive bacteria (Bruntner et al., 2005) and Carboxamycin, an antibiotic isolated from extracts of a *Streptomyces* sp., exhibiting antibacterial activity against Gram-positive bacteria (Hohmann et al., 2009b).

1.9.2. Secondary metabolites with anticancer activity

Cancer is a disease that affects thousands of people worldwide and its incidence is increasing over the years. Most anticancer drugs currently used cause side effects by damaging healthy cells and tissues, and for many cancers no effective treatment is available. Marine bacteria play a major role in the production of secondary metabolites with anticancer activity. Examples illustrated in the literature include Salinosporamide A, a novel bicyclic beta-lactone gamma-lactam isolated from *Salinispora tropica*, which shows cytotoxicity in many malignant cell types (Jensen et al., 2007); Caprolactones, an antibiotic isolated from a strain of *Streptomyces* sp. that also shows activity against cancerous cells and low cytotoxicity in normal cells (Stritzke et al., 2004); Streptoanthraquinone A, a polycyclic anthraquinone recovered from *Streptomyces* sp. which has activity against four different glioma cell lines (Liang et al., 2016); Dehydrorabelomycin and Phenanthroviridone produced by *Micromonospora* sp. and showing varying degrees of cytotoxicity against several cancerous cell lines (Mullowney et al., 2015).

1.10. Aim and outline of this thesis

The search for new drugs to combat diseases for which treatments are inexistent or no

longer effective is an urgent topic. Natural products from marine actinobacteria have been explored to meet this urgent need for new drugs. In this context, the present master's thesis aimed at the isolation of actinobacteria from marine environments, namely from marine sediment and a sponge belonging to the species *Hymeniacidon perlevis*, and investigation of their potential to produce bioactive compounds with antimicrobial or anticancer activities.

The thesis is organized in 4 chapters. Chapter 1 consists in a general introduction to theme of the thesis, where several aspects related with actinobacteria are described, mainly focusing in marine actinobacteria, their environmental distribution, most relevant genera, secondary metabolism and main metabolites produced with antibacterial and anticancer activities. Chapter 2 describes a study on the isolation of actinobacteria from marine sediment collected from the northern Portuguese coast and investigation of their potential to produce compounds with antimicrobial or anticancer activity. Chapter 3, focus on the isolation and identification of rare actinobacteria from the marine sponge *H. perlevis*, obtained from the intertidal zone of the northern Portuguese coast and Chapter 4 consists in a general discussion and main conclusions of the work presented in this thesis.

CHAPTER 2

ISOLATION AND INVESTIGATION OF BIOACTIVE POTENTIAL OF ACTINOBACTERIA FROM PORTUGUESE MARINE COASTAL SEDIMENT

1. Introduction

Multidrug-resistant bacterial infections and cancer are at the top of diseases for which novel therapies are required. The increase of antibiotic resistant microorganisms is mainly caused by the extensive and uncontrolled use of antibiotics, transfer of genes between species and microbial evolution (Duncan et al., 2015). This problem compromises the efficacy of currently available antibiotics, leading to a growing need of finding new therapeutic agents with different mechanisms of action (Undabarrena et al., 2016). On the other hand, most anticancer drugs currently used to treat several types of cancer diseases cause undesirable side effects, damaging healthy cells and tissues while killing cancer cells, and for many types of cancer there is still no effective treatment available, being urgent to discover new molecules with novel anticancer actions and less side effects (Duncan et al., 2015) .

Natural products represent a large and diverse family of chemicals with different biological properties (Katz & Baltz, 2016). Currently, about 34,000 natural products have been characterized and, although they have different origins, the highest percentage of these is produced by bacteria (Berdy, 2012). In the last decades, more than 50% of all approved drugs were from natural origin or inspired in natural molecules, mainly derived from terrestrial organisms (Berdy, 2012; Newman & Cragg, 2016; Spainhour, 2005).

The phylum Actinobacteria constitutes a group of Gram-positive bacteria with diverse physiological and morphological characteristics, usually sharing a high genomic content of guanine+cytosine (Chen et al., 2016). Microorganisms belonging to this phylum are important constituents of microbial communities of terrestrial and marine environments, contributing to the decomposition of organic compounds, nutrient recycling, nitrogen fixation, among others (Barka et al., 2016; Das et al., 2007; Manivasagan et al., 2013). In addition to their important environmental role, these microorganisms, and especially the ones belonging to the class Actinobacteria (hereinafter referred as actinobacteria), are very proficuous in the production of bioactive compounds with high pharmaceutical and industrial interest, being responsible for the producing of about 40% (*ca.* 13,700) of all bioactive microbial metabolites known. Among these, *Streptomyces* species are outstanding producers of bioactive compounds, producing *ca*. 76% of all the microbial metabolites derived from actinobacteria (e.g., streptomycin, rapamycin and tetracycline) (Berdy, 2005;2012; Manivasagan et al., 2013). Nonetheless, rare actinobacteria such as *Micromonospora*, *Actinomadura*, *Actinoplanes*, *Streptoverticillium*, *Nocardia,*

Saccharopolyspora and *Streptosporangium,* have also revealed to be important producers of bioactive compounds (Berdy, 2005; 2012).

Microorganisms producing natural compounds have been intensively searched in terrestrial environments, leading to an overexploitation of these ecosystems and to a reduction in the chances of finding new bioactive molecules (Manivasagan et al., 2014). In the last years, a high interest in marine microorganisms has emerged not only for their taxonomic diversity and ecological importance, but also for their potential to produce new natural products (Dharmaraj, 2010). Oceans are home for a great diversity of microorganisms though a large majority of marine ecosystems is still unexplored in terms of actinobacterial diversity and, therefore, of new metabolites (Manivasagan et al., 2014). In fact, the indigenous character of marine actinobacteria has only been recently recognized and these microorganisms have proven to be an important source of bioactive compounds. Examples include marinomycins, produced by a *Marinospora* sp. and exhibiting antitumor and antibacterial activity; salinosporamide, resultant from the species *Salinispora tropica* and a potent anticancer agent and the antibiotic abyssomicin produced by *Verrucosispora maris* (Manivasagan et al., 2013;2014; Subramani & Aalbersberg, 2012; Ward & Bora, 2006).

Marine sediments of the Atlantic Ocean are very poorly explored (Prieto-Davó et al., 2016) and this includes the vast maritime territory of Portugal for which the associated actinobacterial population is scarcely studied. Such underexplored environments offer an excellent opportunity to access novel microorganisms, thus increasing the prospects of finding new natural compounds with bioactivity (Claverias et al., 2015).

In this study, a sediment sample collected from the intertidal zone of a beach located in Parque Natural do Litoral Norte, in northern Portugal, was used to investigate the associated actinobacteria and their bioactivity potential, through a cultivation-dependent approach. The Parque Natural do Litoral Norte is a protected area of Portugal that extends along 16 km of coast. This site was chosen because it has minimal anthropogenic intervention, with the inherent microbial communities being almost exclusively affected by natural processes. The main objectives of this study were to isolate and identify the actinobacterial population associated with the collected marine sediment and to investigate their ability to produce bioactive compounds with antimicrobial or anticancer activities.

2. Materials and Methods

2.1. Sample collection

A sample of marine sediment was collected at a depth of 0.15 m in the intertidal zone of Cepães beach located in Parque Natural do Litoral Norte, in the coastal area of Northern Portugal (41°33'11.5"N 8°47'38.1"W). The sample was transferred to a sterile plastic bag, transported to the laboratory in a cooling box and processed within an interval of two hours.

2.2. Isolation of actinobacteria

Before inoculation onto isolation media, the sediment sample was processed using three different methods: dilution of 1 g of sediment in 9 mL of filtered seawater (method 1); dilution of 1 g of sediment in 9 mL of filtered seawater followed by incubation in a water bath at 60 ºC for 10 min (method 2); dilution of 5 g of sediment in 15 mL of filtered seawater and incubation with 20 mgL^{-1} of nalidixic acid and 20 mgL^{-1} of cycloheximide, at 28 °C for 30 min (method 3). Samples resulting from these three methods were ten-fold diluted to 10^{-5} and an aliquot of 100 µl of each dilution was spread over the surface of the following selective isolation media: M3 agar (per liter of distilled water): 0.466 g of KH_2P0_4, 0.732 g of Na_2HPO_4, 0.10 g of KNO_3, 0.29 g of NaCl, 0.10 g of $MgSO_4.7H_2O$, 0.02 g of $CaCO_3$, 200 µg of $FeSO_4.7H_20$, 180 µg of $ZnSO_4.7H_2O$, 15 µg of $MnSO_4.4H_20$, 4 mg of thiamine HCl (Vitamin B1) and 17 g of agar; Nutrient-poor sediment extract (NPS) (per liter of seawater): 100 mL of marine sediment extract (obtained by washing 900 mL of sediments with 500 mL of seawater) and 17 g of agar; Starch-casein-nitrate agar (SNC) (per liter of distilled water): 10 g of soluble starch, 0.3 g of casein, 2 g of K_2HPO_4, 2 g of KNO_3, 2 g of NaCl, 0.05 g of $MgSO_4.7H_2O$, 0.02 g of $CaCO_3$, 0.01 g of $FeSO_4.7H_20$ and 17 g of agar. All media were supplemented with cycloheximide (50 mgL^{-1}) and streptomycin (50 mgL^{-1}) in order to inhibit the growth of fungi and Gram-negative bacteria. The plates were incubated for six weeks at a temperature of 28 °C.

Along the incubation period, plates were periodically inspected by visual observation and all colonies with different morphological characteristics were picked and re-streaked numerous times in the same agar medium until obtainment of pure colonies. Each isolate

was cryopreserved at -80 ºC in 30% glycerol. Before the cryopreservation step, each isolate was grown in liquid ISP2 medium or in Marine broth (Laboratorios Conda, S.A) in order to obtain enough biomass for cryopreservation (Table 2). The ISP2 medium had the following composition (per liter of seawater): 4 g of yeast extract, 10 g of malt extract, 4 g of glucose and 17 g of agar. The two media used were supplemented with the same antibiotics described above.

2.3. Identification of isolated strains by 16S rRNA gene sequencing

Genomic DNA was extracted from all isolated microorganisms though the phenol-chloroform method, as described elsewhere (Pereira et al., 2010). To obtain biomass for DNA extraction, each isolate was grown in 5 mL of liquid ISP2 medium or Marine broth (Table S1), at 25 ºC, 100 rpm, for one week. Biomass pellet was obtained for each isolate by centrifugation of 2 ml broth (7,000 x g for 5 min).

16S rRNA gene was amplified by PCR, using the universal primers 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-30') (Lane, 1991). The PCR mixture (total volume of 10 µL) contained: 5 µL of Taq PCR Master Mix (Qiagen, Valencia, CA), 1 µL of primer 27F (2 µM), 1 µL of primer 1492R (2 µM) and 3 µL of DNA template. PCR conditions were as following: initial denaturation at 95 °C for 15 min, followed by 30 cycles at 94 °C for 30 s, 48 °C for 90 s, 72 °C for 90 s and a final extension at 72 °C for 10 min. PCR products were separated on a 1.5% agarose gel containing SYBR Safe (ThermoFisher Scientific, USA), at 150 V for 30 min. Purification and sequencing of the amplified DNA was performed by GATC Biotech (European Genome and Diagnostics Centre, Constance, Germany). The obtained 16S rRNA sequences were analyzed using the Geneious software, version 4.8.2 and the resulting consensus sequences were compared to the GenBank database using the blastn algorithm. The consensus sequences were additionally compared with the databases EzTaxon (http://www.ezbiocloud.net/) and Ribosomal Database Project (https://rdp.cme.msu.edu/index.jsp) in order to confirm the results obtained in the GenBank database.

All the sequences and their five closest neighbor sequences in Genbank were aligned using the Geneious software. Phylogenetic trees were made individually for each phylum.

All alignments were used to generate a Maximum Likelihood phylogenetic tree of 1112bp for the phylum Actinobacteria, 1045bp for the phylum Firmicutes, 1365bp for phylum Proteobacteria and 1237bp for phylum Bacteroidetes, performed as the defaults of the ATCG South of France bioinformatics platform (http://www.atgc-montpellier.fr/) with 100 bootstraps

Sequences of MS8A1, MS40A, MS50 and MS58 strains belonging to the actinobacteria phylum and sequences of the MS7J, MS10G, MS13, MS22A, MS26F and MS57 strains belonging to the Firmicutes phylum were not included in the trees because they had sequences of <900bp.

2.4. Preparation of crude extracts from actinobacterial cultures for bioactivity tests

In order to test the bioactivity potential, namely antimicrobial and anticancer activities, of the isolated actinobacterial strains, each isolate was grown in 250 mL Erlenmeyer flasks containing 100 mL of ISP2 medium or Marine broth (Laboratorios Conda, S.A) (Table 2). The flasks were incubated at 25 ºC, 100 rpm, in the dark. Cultures were grown for 1-2 weeks, depending on their growth rate, after which 1.5 g Amberlite® XAD16N resin (Sigma-Aldrich, St. Louis, Mo.) were added to the cultures and left to incubate for an additional period of one week. Cultures were extracted twice with a solution of methanol/acetone 1:1 ratio (v/v). The organic layer was dried in a rotary evaporator and the resulting extract was dissolved in DMSO to obtain stock solutions with final concentrations of 3 mg mL^{-1} and 1 mg mL^{-1}, to be tested in the bioactivity assays.

2.5. Anticancer Bioactivity Screening

The actinobacterial extracts were tested against two cancer cell lines: breast ductal carcinoma (T-47D) and neuroblastoma (SH-SY5Y), both from Sigma-Aldrich (St. Louis, Missouri, USA). Cells were grown in Dubelco's Modified Eagle Medium (DMEM) from Gibco (Thermo Fischer Scientific, Waltham, Massachusetts, USA) supplemented with 10% (v/v) fetal bovine serum (Biochrom, Berlin, Germany), 1% (v/v) penincillin/streptomycin (Biochrom) at 100 IU/mL and 10 mg/mL, respectively, and 0.1% (v/v) amphotericin (GE

Healthcare, Little Chafont, United Kingdom) The cells were incubated at 37 °C in a humidified atmosphere containing 5% of CO_2.

Cell viability was assessed by the MTT assay ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The cells were seeded in 96-well plates at a density of 6.6×10^4 cells mL^{-1}. After 24 h, cells were exposed to the extracts at a final concentration 30 µg mL^{-1}. Negative and positive controls consisted in 0.5% dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) and 20% DMSO, respectively. Cell viability was assed at 24 and 48 h by adding MTT at a final concentration of 0.2 mg mL^{-1} and incubating for 4 h at 37 °C. The medium was then removed and 100 µL of DMSO were added per well, after which the absorbance was read at 570 nm (Synergy HT, Biotek, USA). Cellular viability was expressed as a percentage relative to the negative control. The assays were performed in triplicate.

2.6. Antimicrobial bioactivity screening

The screening of antimicrobial activity in the obtained actinobacterial extracts was performed using the agar-based disk diffusion method. The extracts were tested against 5 reference microorganisms: *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 25241) and *Candida albicans* (ATCC 10231). The bacterial strains were grown in Mueller-Hinton agar (MH; BioKar diagnostics) and the yeast, *C. albicans*, was grown in Sabouraud Dextrose agar (SD; BioKar diagnostics). Colonies of test organisms were suspended in the corresponding media broth and the turbidity of the cultures was adjusted to 0.5 McFarland standard (OD_{625}= 0.08−0.13). These standardized suspensions were used to seed MH or SD (for *C. albicans*) agar plates, by evenly streaking the plates with a swab dipped in the inoculum cultures. Blank paper discs (6mm in diameter; Oxoid) were placed in the surface of the inoculated plates and loaded with 15 µl of each extract at the concentration of 1 mg mL^{-1}. Negative controls consisted in loading the paper disks with 15 µl of DMSO at 1 mg mL^{-1}. The assays were performed in duplicate. Agar plates were incubated at 37°C for 24 h, after which the presence of inhibition zone diameters was inspected. Minimal inhibitory concentration (MIC) was only determined for extracts showing inhibition halos. Inoculum suspension of the reference microorganism *C. albicans* was prepared as above. Stock solutions of extracts were prepared in the medium broth SD at the concentration of 500 µg

mL^{-1} and ten two-fold dilutions were successively performed from these stocks, resulting in extracts concentrations ranging from 250 to 0.487 µg mL^{-1}. The assay was performed in 96 well plates, in which each well was filled with 50 µL of microbial inoculum (diluted 1:100) and 50 µL of each extract dilution. Positive control consisted in filling the wells with 50 µL of microbial inoculum and 50 µL of medium broth and negative controls consisted in filling the wells with 100 µL of medium broth. Dilutions of each extract were tested in triplicate. MIC was determined by spectrophotometric analysis (at 625 nm), after 18 h of incubation at 37 °C.

3. Results

The isolation of actinobacteria from a marine sediment sample collected on a beach situated in Parque Natural do Litoral Norte, Portugal, was carried out using three different treatments and three selective culture media. A total of 117 isolates with distinct morphological characteristics were obtained along an incubation period of six weeks. Many of these isolates exhibited traits characteristic of actinobacteria, such as colonies with a leather texture, growing inside the agar, slow growth, production of spores or hyphae and production of pigments leading in some cases to change of the color of the medium (Fig 3).

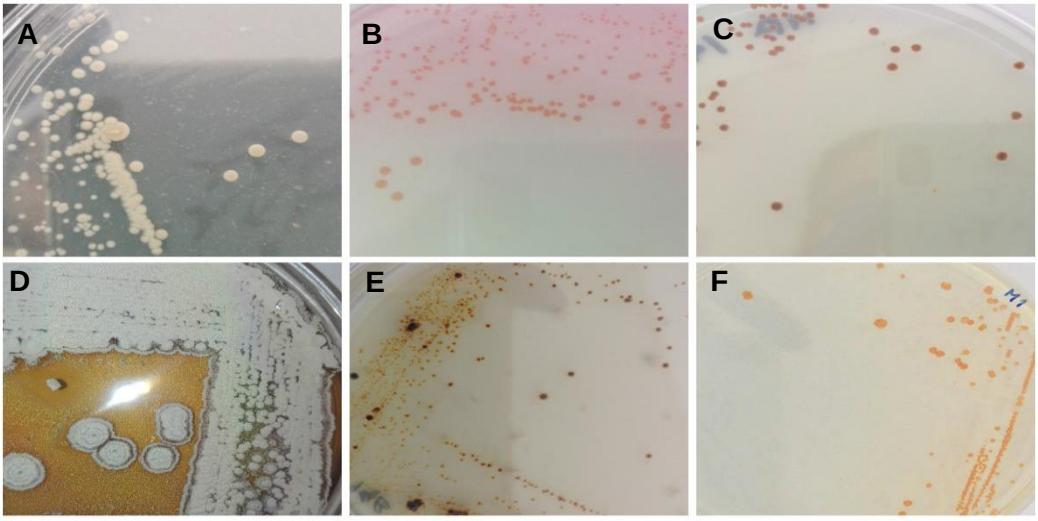

Figure 3 - Examples of some isolates obtained from marine sediment collected from a beach in Parque Natural do Litoral Norte. (A) Strain MS58, (B) strain MS55, (C) strain MS8B, (D) strain MS35, (E) strain MS14B, and (F) strain MS3C.

Taxonomic identification of all obtained isolates revealed that the treatments and culture media used in this study led to the isolation of microorganisms belonging to 4 different phyla: Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes (Fig 4 and Fig 5, Fig 6, Fig 7 and Fig 8). The results of the identification of each isolate through 16S rRNA gene sequencing are indicated in Table 2 and the distribution of the identified genera according to their abundance, treatment methods employed and culture media used is shown in Figure 9.

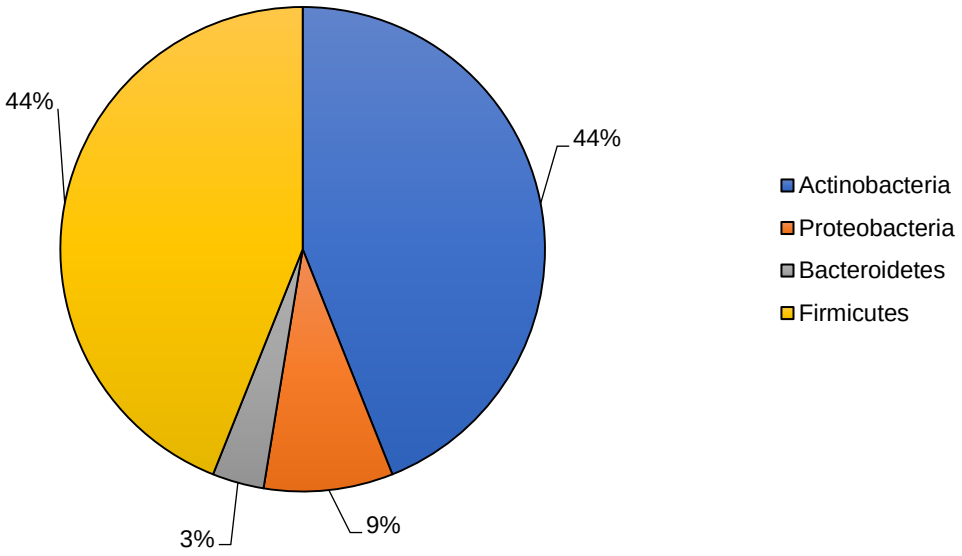

Figure 4 – Distribution by phylum of the isolates recovered from the marine sediment collected on Parque Natural do Litoral Norte, Portugal.

Within the phylum Actinobacteria, which was the focus of this study, the results revealed that the recovered isolates had a high phylogenetic diversity, belonging to 6 different families: *Micromonosporaceae*, *Streptomycetaceae*, *Micrococcaceae*, *Microbacteriaceae*, *Nocardiopsaceae* and *Thermomonosporaceae* and 7 genera: *Micromonospora*, *Polymorphospora*, *Streptomyces*, *Arthrobacter*, *Herbiconiux*, *Nocardiopsis* and *Actinomadura*, with the largest fraction of isolates being assigned to the genus *Micromonospora* (Fig. 9A). Interestingly, except for the genera *Streptomyces*, *Arthrobacter* and *Herbiconiux* all the other genera constitute rare actinobacterial genera. From the analysis of Fig. 9, it is also apparent that the three methods used to treat the marine

sediment sample allowed a similar recovery of *Micromonospora* species, while a higher number of Streptomyces isolates was obtained with methods 1 and 2 (Fig. 9A2). It is notable that the vast majority of the actinobacterial isolates obtained were recovered from the medium NPS, with SCN retrieving a small fraction of actinobacteria and M3 medium showing no growth. (Fig. 9A3).

Concerning the phyla Firmicutes, Proteobacteria and Bacteroides, isolates belonging to the first phylum were assigned to only two genera: *Bacillus* and *Paenibacillus*, (Table 2 and Fig. 9B1), while for the Proteobacteria and Bacteroides phyla the few isolates recovered were found to: belong to 4 (*Erythrobacter*, *Alterytrobacter*, *Devosia* and *Sphingorhabdus*) and 3 (*Leeuwenhokiella*, *Flexibacter* and *Maribacter*) different genera, respectively (Table 2 and Figs. 9C1 and 9D1). The three treatments applied in the marine sediment sample did not seem to have influenced the recovery of *Bacillus* and *Paenibacillus* species, while these treatments appeared to have some influence in the recovery of the different genera belonging to the Proteobacteria and Bacteroides phyla (Fig. 9B2, 9C2 and 9D2). The medium that led to a higher number of Firmicutes, Proteobacteria and Bacteroides isolates was, once again, the NPS. Medium SCN only allowed the recovery of a few Bacillus species, while no isolates were grown in medium M3 (Fig. 9B3, 9C3 and 9D3).

Table 2 - Taxonomic identification of the isolates recovered from the marine sediment collected from a beach in Parque Natural do Litoral Norte. Different colors in the Table refer to microorganisms belonging to different phyla.

Isolate	Sample[a] processing method	Isolation Medium	Liquid medium used for growth	Phylum	Closest identification	Similarity %
MS3D1	Method 1	NPS	Marine Broth	Actinobacteria	*Streptomyces sp*	100
MS5D	Method 1	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.3
MS5E	Method 1	NPS	Marine Broth	Actinobacteria	*Micromonospora aurantiaca*	100
MS19E	Method 1	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.8
MS19F	Method 1	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.8
MS23C	Method 1	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.0
MS23D	Method 1	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.8
MS26H	Method 1	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.5
MS26I	Method 1	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.5
MS32	Method 1	NPS	ISP2	Actinobacteria	*Streptomyces zhaozhouensis*	99.9
MS35	Method 1	NPS	ISP2	Actinobacteria	*Nocardiopsis* sp	99.9
MS42	Method 1	NPS	Marine Broth	Actinobacteria	*Micromonospora aurantiaca*	100
MS46	Method 1	NPS	Marine Broth	Actinobacteria	*Micromonospora peceutia*	99.6
MS48	Method 1	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.8
MS49	Method 1	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.9
MS50	Method 1	NPS	Marine Broth	Actinobacteria	*Micromonospora peucetia*	99.4
MS51	Method 1	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.8
MS54	Method 1	NPS	ISP2	Actinobacteria	*Streptomyces* sp.	100
MS3B	Method 2	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	100
MS3C1	Method 2	NPS	Marine Broth	Actinobacteria	*Micromonospora marina*	99.8
MS16B	Method 2	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.8

MS18A	Method 2	NPS	ISP2	Actinobacteria	*Streptomyces* sp	99.6
MS18B	Method 2	NPS	Marine Broth	Actinobacteria	*Streptomyces* sp	100
MS19	Method 2	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.2
MS19A	Method 2	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.9
MS19B1	Method 2	NPS	Marine Broth	Actinobacteria	*Micromonospora aurantiaca*	100
MS19C	Method 2	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.7
MS19D	Method 2	NPS	Marine Broth	Actinobacteria	*Micromonospora marina*	99.6
MS23A	Method 2	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.6
MS23B	Method 2	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.7
MS24	Method 2	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.6
MS27A	Method 2	NPS	ISP2	Actinobacteria	*Streptomyces* sp	99.9
MS29	Method 2	NPS	ISP2	Actinobacteria	*Streptomyces* sp	100
MS38	Method 2	SCN	ISP2	Actinobacteria	*Herbiconiux solani*	100
MS52A	Method 2	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.6
MS52B	Method 2	NPS	Marine Broth	Actinobacteria	*Micromonospora marina*	99.0
MS53	Method 2	NPS	Marine Broth	Actinobacteria	*Polymorphospora rubra*	99.6
MS3C	Method 3	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.8
MS3E	Method 3	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.7
MS5B	Method 3	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.6
MS5C	Method 3	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.6
MS8A1	Method 3	NPS	ISP2	Actinobacteria	*Streptomyces* sp.	99.7
MS8B	Method 3	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp	99.3
MS8C	Method 3	NPS	Marine Broth	Actinobacteria	*Micromonospora* sp.	99.7
MS14B	Method 3	NPS	Marine Broth	Actinobacteria	*Streptomyces* sp.	98.2
MS39B	Method 3	SCN	ISP2	Actinobacteria	*Arthrobacter* sp	99.8

MS39C	Method 3	SCN	ISP2	Actinobacteria	*Arthrobacter* sp	99.8
MS40A	Method 3	SCN	ISP2	Actinobacteria	*Arthrobacter* sp	100
MS40B1	Method 3	SCN	ISP2	Actinobacteria	*Arthrobacter* sp	99.8
MS55	Method 3	NPS	Marine Broth	Actinobacteria	*Micromonospora aurantiaca*	100
MS56	Method 3	NPS	Marine Broth	Actinobacteria	*Micromonospora aurantiaca*	99.6
MS58	Method 3	NPS	Marine Broth	Actinobacteria	*Actinomadura sputi*	99.6
MS2I	Method 1	NPS	ISP2	Firmicutes	*Bacillus* sp.	99.3
MS3D2	Method 1	NPS	ISP2	Firmicutes	*Bacillus* sp.	99.7
MS3H	Method 1	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	99.2
MS7N	Method 1	NPS	ISP2	Firmicutes	*Bacillus* sp.	99.3
MS7S	Method 1	NPS	ISP2	Firmicutes	*Bacillus* sp.	99.6
MS7T2	Method 1	NPS	ISP2	Firmicutes	*Bacillus licheniformis*	99.8
MS15B	Method 1	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	98.7
MS23G	Method 1	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	98.6
MS26D	Method 1	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	97.0
MS26F	Method 1	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	99.3
MS26K	Method 1	NPS	ISP2	Firmicutes	*Paenibacillus agarexendens*	99.0
MS31B	Method 1	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	98.4
MS33	Method 1	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	98.9
MS45	Method 1	NPS	ISP2	Firmicutes	*Bacillus* sp.	99.5
MS7J	Method 2	NPS	ISP2	Firmicutes	*Bacillus licheniformis*	100
MS7K	Method 2	NPS	ISP2	Firmicutes	*Bacillus* sp.	99.8
MS7L	Method 2	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	98.0
MS7M	Method 2	NPS	ISP2	Firmicutes	*Bacillus* sp.	99.4
MS7P	Method 2	NPS	ISP2	Firmicutes	*Bacillus* sp.	99.5

MS7O	Method 2	NPS	ISP2	Firmicutes	*Bacillus licheniformis*	99.4
MS7Q	Method 2	NPS	ISP2	Firmicutes	*Bacillus* sp.	99.4
MS7U	Method 2	NPS	ISP2	Firmicutes	*Bacillus licheniformis*	99.5
MS10D	Method 2	NPS	ISP2	Firmicutes	*Paenibacillus amylolyticus*	99.1
MS10G	Method 2	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	100
MS10H	Method 2	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	99.2
MS22A	Method 2	NPS	ISP2	Firmicutes	*Bacillus licheniformis*	99.7
MS25B	Method 2	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	99.6
MS26B1	Method 2	NPS	ISP2	Firmicutes	*Paenibacillus daejenonensis*	99.4
MS26C	Method 2	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	99.4
MS27A	Method 2	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	99.8
MS28A	Method 2	NPS	ISP2	Firmicutes	*Bacillus* sp.	99.7
MS2C	Method 3	NPS	ISP2	Firmicutes	*Paenibacillus xylanilyticus*	98.8
MS2D	Method 3	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	99.4
SM2E	Method 3	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	98.4
SM2F	Method 3	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	99.3
MS6	Method 3	NPS	ISP2	Firmicutes	*Paenibacillus agarexendens*	99.2
MS7A	Method 3	NPS	ISP2	Firmicutes	*Bacillus licheniformis*	99.8
MS7B	Method 3	NPS	ISP2	Firmicutes	*Bacillus licheniformis*	99.7
MS7C	Method 3	NPS	ISP2	Firmicutes	*Bacillus* sp.	99.7
MS7D	Method 3	NPS	ISP2	Firmicutes	*Bacillus* sp.	99.7
MS7E	Method 3	NPS	ISP2	Firmicutes	*Bacillus* sp.	99.8
MS7I	Method 3	NPS	ISP2	Firmicutes	*Bacillus* sp.	99.4
MS7V	Method 3	NPS	ISP2	Firmicutes	*Bacillus* sp.	99.4
MS8A2	Method 3	NPS	ISP2	Firmicutes	*Bacillus paralicheniformis*	99.5

MS11A	Method 3	NPS	ISP2	Firmicutes	*Paenibacillus xylanexedens*	99.7
MS12A	Method 3	NPS	ISP2	Firmicutes	*Paenibacillus* sp.	99.2
MS13	Method 3	NPS	ISP2	Firmicutes	*Bacillus siralis*	99.5
MS14A	Method 3	NPS	ISP2	Firmicutes	*Bacillus paralicheniformis*	99.8
MS15A	Method 3	NPS	ISP2	Firmicutes	*Paenibacillus* sp..	99.0
MS39A	Method 3	SCN	ISP2	Firmicutes	*Bacillus* sp.	99.7
MS57	Method 3	NPS	ISP2	Firmicutes	*Bacillus clausii*	99.5
MS43	Method 1	NPS	NPS	Proteobacteria	*Devosia* sp.	98.4
MS20C	Method 2	NPS	NPS	Proteobacteria	*Sphingorhabdus flavimaris*	99.6
MS21A	Method 2	NPS	NPS	Proteobacteria	*Erythrobacter pelagi*	99.4
MS2C2	Method 3	NPS	NPS	Proteobacteria	*Erythrobacter* sp.	100
MS2E 1	Method 3	NPS	NPS	Proteobacteria	*Altererythrobacter* sp.	97.9
MS3A	Method 3	NPS	NPS	Proteobacteria	*Erythrobacter longus*	98.9
MS4B	Method 3	NPS	NPS	Proteobacteria	*Altererythrobacter dongtanensis*	99.5
MS4C	Method 3	NPS	NPS	Proteobacteria	*Erythrobacter* sp.	97.7
MS5A	Method 3	NPS	NPS	Proteobacteria	*Altererythrobacter* sp.	97.8
MS11E	Method 3	NPS	NPS	Proteobacteria	*Erythrobacter* sp.	99.1
MS1E	Method 1	NPS	NPS	Bacteroidetes	*Leeuwenhoekiella* sp.	98.9
MS30	Method 1	NPS	NPS	Bacteroidetes	*Maribacter* sp.	98.0
MS1D	Method 2	NPS	NPS	Bacteroidetes	*Leeuwenhoekiella* sp.	99.0
MS20B	Method 2	NPS	NPS	Bacteroidetes	*Fluvivirga sp*	96.0

[a]Method 1: dilution of 1 g of sediment in 9 mL of filtered seawater; Method 2: dilution of 1 g of sediment in 9 mL of filtered seawater followed by incubation in a water bath at 60 ºC for 10 min; Method 3: dilution of 5 g of sediment in 15 mL of filtered seawater and incubation with 20 mgL^{-1} of nalidixic acid and 20 mgL^{-1} of cycloheximide, at 28 °C for 30 min.

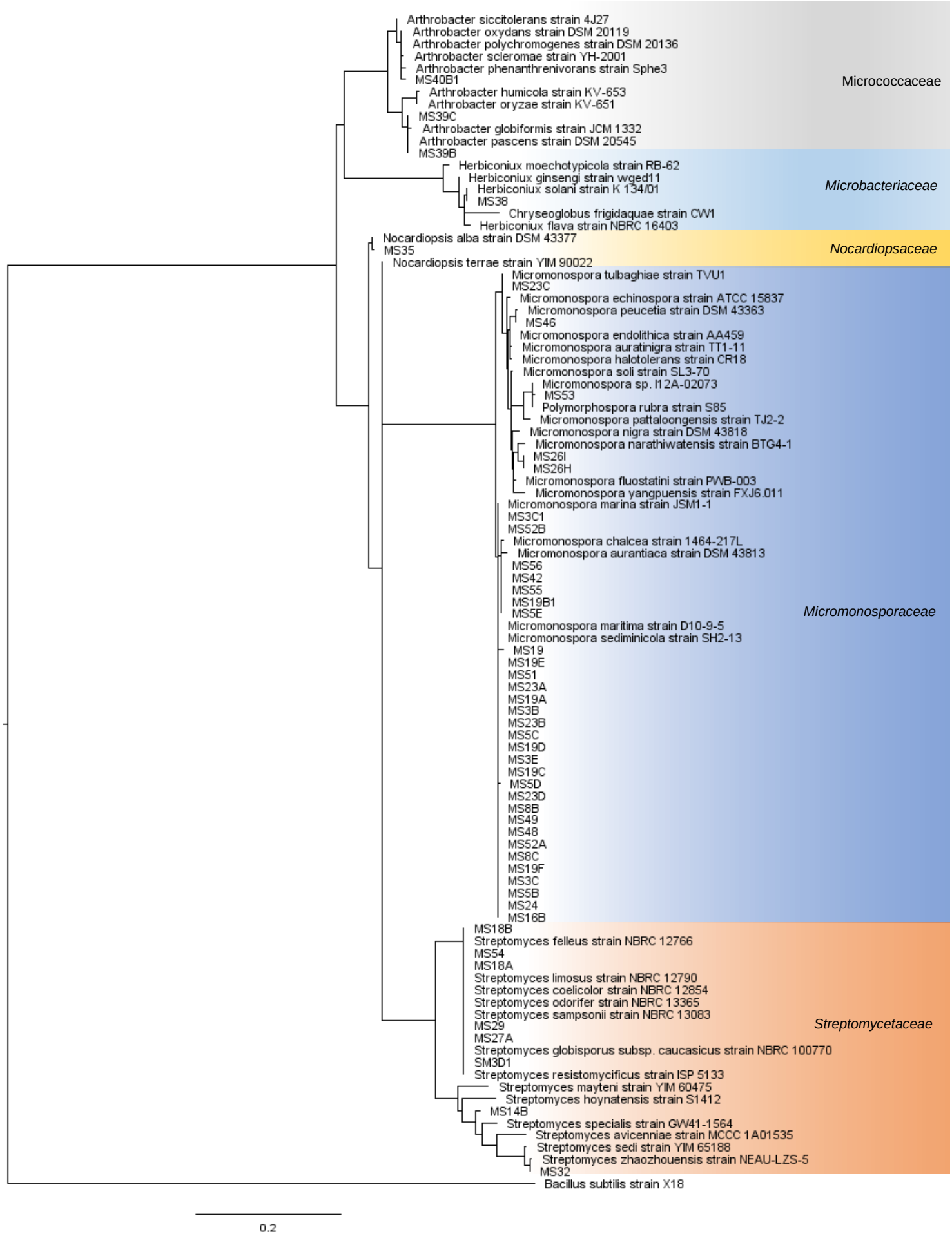

Figure 5 - **Phylogenetic tree of strains belonging to the phylum Actinobacteria, isolated from marine sediments, and their GenBank nearest neighbor**. Maximum Likelihood phylogenetic tree was performed using 99 sequences with 1112bp and 100 bootstraps. *Bacillus subtilis* was used as an outgroup.

Figure 6 - **Phylogenetic tree of strains belonging to the phyla Firmicutes isolated from marine sediments, and their GenBank nearest neighbor**. Maximum Likelihood phylogenetic tree was performed using 90 sequences with 1045bp and 100 bootstraps *Escherichia coli* was used as an outgroup.

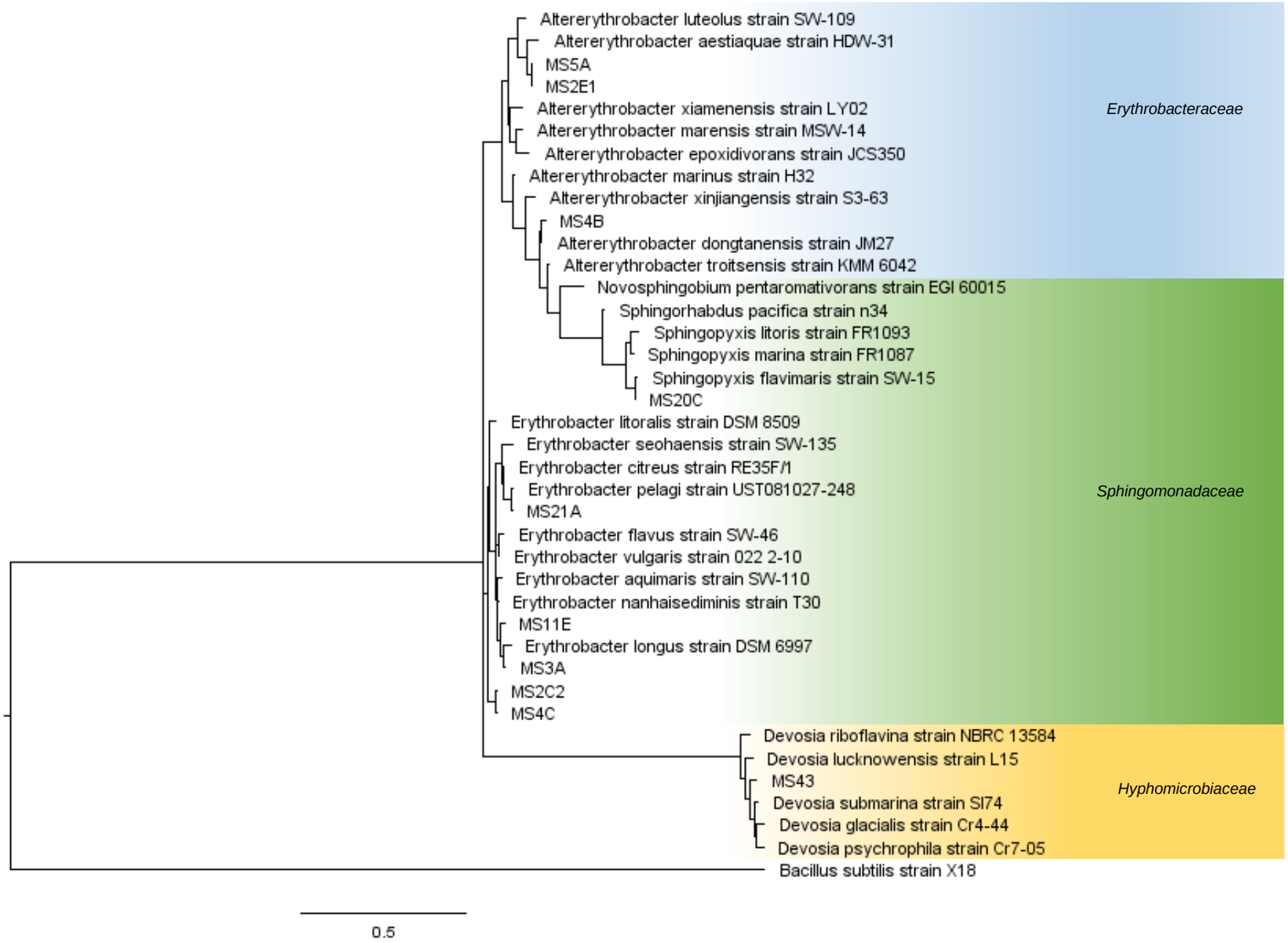

Figure 7 - **Phylogenetic tree of strains belonging to the phyla Proteobacteria, isolated from marine sediments, and their GenBank nearest neighbor**. Maximum Likelihood phylogenetic tree was performed using 40 sequences with 1365bp and 100 bootstraps. *Bacillus subtilis* was used as an outgroup.

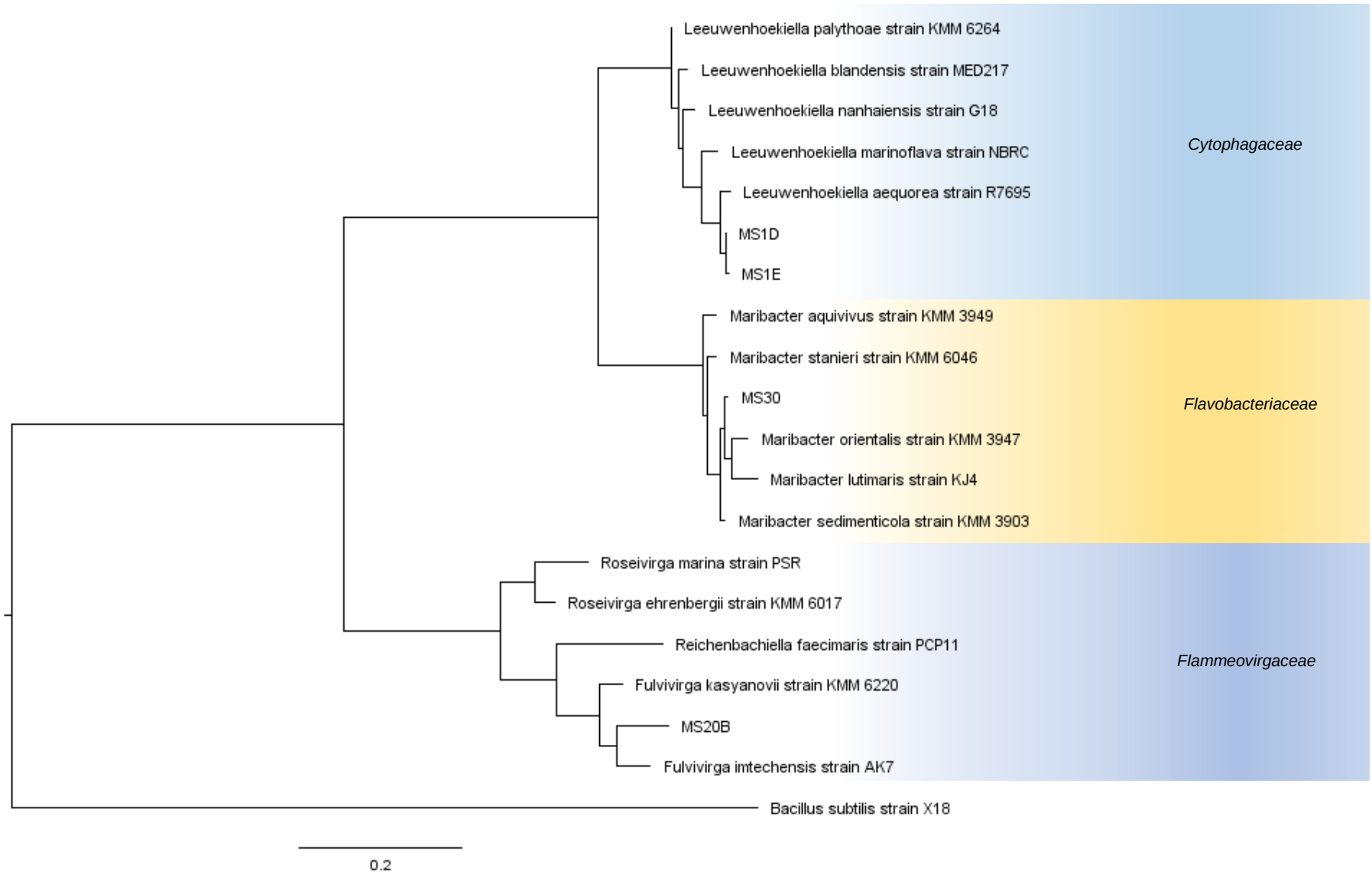

Figure 8 - **Phylogenetic tree of strains belonging to the phyla Bacteroidetes, isolated from marine sediments, and their GenBank nearest neighbor**. Maximum Likelihood phylogenetic tree was performed using 20 sequences with 1237bp and 100 bootstraps. *Bacillus subtilis* was used as an outgroup.

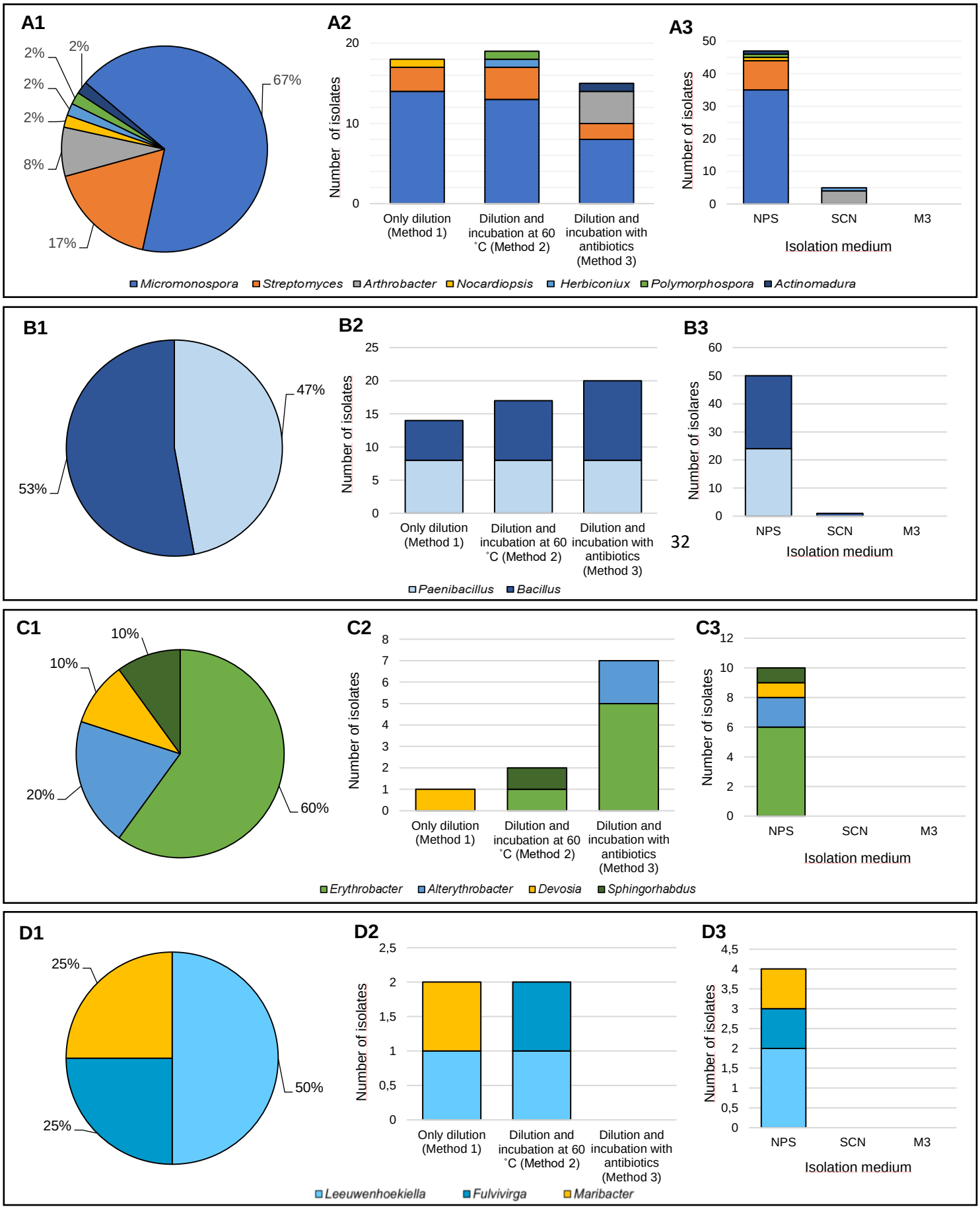

Figure 9- Distribution, in terms of genera, of the isolates recovered from the marine sediment collected on Parque Natural do Litoral Norte, according to abundance, treatment methods and selective culture media used. Percentage of isolates belonging to indicated genera within the phylum Actinobacteria (A1), Firmicutes (B1), Proteobacteria (C1) and Bacteroidetes (D1); Number of isolates belonging to indicated genera recovered according to the culture media used within the phylum Actinobacteria (A2), Firmicutes (B2), Proteobacteria (C2) and Bacteroidetes (D2); Number of isolates belonging to indicated genera recovered according to the treatment methods used within the phylum Actinobacteria (A3), Firmicutes (B3), Proteobacteria (C3) and Bacteroidetes (D3).

The bioactivity potential of all actinobacterial strains isolated in this study was investigated by screening crude extracts obtained from these isolates in terms of their antimicrobial activity against 5 reference strains - *S. aureus, B. subtilis, E. coli, S. typhimurium* and *C. albicans* and anticancer activity in the cell lines T47-D breast ductal carcinoma and SH-SY5Y neuroblastoma.

The results presented in Table 3 show that crude extracts of 6 isolates belonging to the genus Streptomyces (strains MS8A1, MS18A, MS18B, MS27A, MS29 and MS54) exhibit antimicrobial activity against *C. albicans* (Fig. 10). Determination of MIC for these crude extracts revealed a biological activity against *C. albicans* in the range of 125-3.90 µl mL^{-1}, with strain MS18B presenting the lowest MIC value (3.90 µl mL^{-1}).

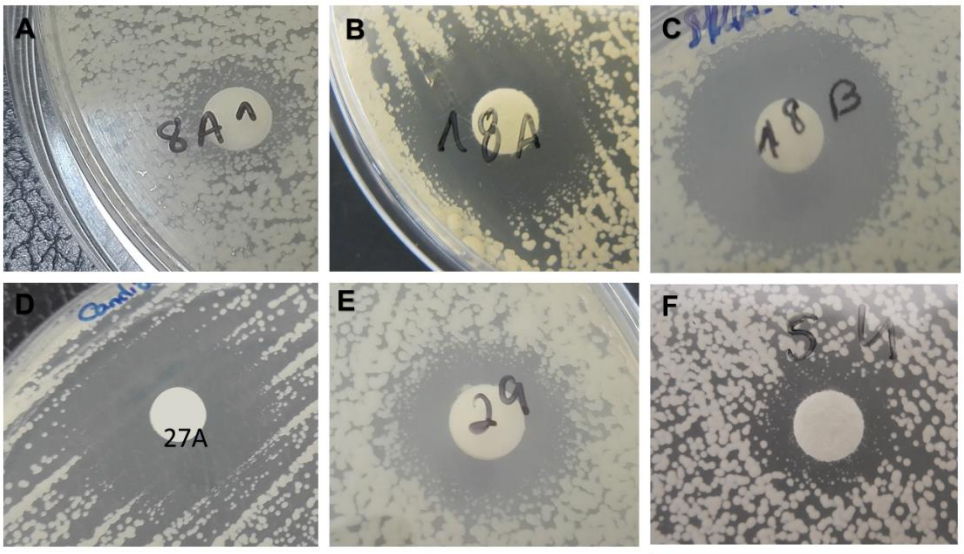

Figure 10 -Inhibition zones caused in *Candida albicans* by crude extracts of the strains MS8A1 (A), MS18A (B), MS18B (C) MS27A (D), MS29 (E) and MS54 (F).

For the investigation of anticancer activity, the % of cell viability after exposure to actinobacterial crude extracts for 24 h and 48 h was measured in two cell lines. The final concentration of actinobacterial crude extracts in the MTT assay was 15 µg mL^{-1}. The results showed that, after 48 h of exposure, crude extracts of 3 actinobacterial isolates presented cytotoxic activity against at least one the cell lines tested. Strains MS3B, MS3E and MS56, all belonging to the genus *Micromonospora*, showed 8%, 5% and 12% of cell viability, after 48 h, in the cell lines T47-D or SH-SY5Y. Strain MS18B (*Streptomyces* sp.) was the one that revealed the best results, presenting a % of cell viability <10 for both cancer cell lines tested.

Table 3 - Antimicrobial and anticancer activities of crude extracts from the actinobacterial isolates obtained in this study

Isolate	Closest identification	Antimicrobial screening assay						Anticancer screening assay (% of cell viability[a])			
		Disk difusion method					MIC (µg mL^{-1})	T47-D		SH-SY5Y	
		S. aureus	*B. subtilis*	*E. coli*	*S. typhimurium*	*C. albicans*	*C. albicans*	24H	48H	24H	48H
MS3B	*Micromonospora* sp.	-	-	-	-	-	NT	≈104	≈8	≈67	≈90
MS3C	*Micromonospora* sp.	-	-	-	-	-	NT	≈98	≈109	≈95	≈55
MS3C1	*Micromonospora marina*	-	-	-	-	-	NT	≈119	≈93	≈98	≈72
MS3D1	*Streptomyces* sp.	-	-	-	-	-	NT	≈73	≈83	≈63	≈62
MS3E	*Micromonospora* sp.	-	-	-	-	-	NT	≈10	≈5	≈9	≈46
MS5B	*Micromonospora* sp.	-	-	-	-	-	NT	≈132	≈96	≈69	≈64
MS5C	*Micromonospora* sp.	-	-	-	-	-	NT	≈82	≈95	≈73	≈96
MS5D	*Micromonospora* sp.	-	-	-	-	-	NT	≈117	≈108	≈107	≈76
MS5E	*Micromonospora aurantiaca*	-	-	-	-	-	NT	≈121	≈99	≈75	≈82
MS8A1	*Streptomyces* sp.	-	-	-	-	+	15.62	≈90	≈86	≈86	≈120
MS8B	*Micromonospora* sp.	-	-	-	-	-	NT	≈85	≈92	≈68	≈130

MS8C	*Micromonospora* sp.	-	-	-	-	-	NT	≈105	≈71	≈94	≈86
MS14B	*Streptomyces* sp.	-	-	-	-	-	NT	≈127	≈105	≈96	≈81
MS16B	*Micromonospora* sp.	-	-	-	-	-	NT	≈112	≈111	≈86	≈85
MS18A	*Streptomyces* sp.	-	-	-	-	+	15.62	≈93	≈88	≈83	≈74
MS18B	*Streptomyces* sp.	-	-	-	-	+	3.90	≈20	≈8	≈1	≈2
MS19	*Micromonospora* sp.	-	-	-	-	-	NT	≈117	≈90	≈85	≈100
MS19A	*Micromonospora* sp.	-	-	-	-	-	NT	≈70	≈98	≈125	≈99
MS19B1	*Micromonospora aurantiaca*	-	-	-	-	-	NT	≈103	≈98	≈105	≈148
MS19C	*Micromonospora* sp.	-	-	-	-	-	NT	≈107	≈102	≈112	≈71
MS19D	*Micromonospora* sp.	-	-	-	-	-	NT	≈116	≈103	≈89	≈91
MS19E	*Micromonospora* sp.	-	-	-	-	-	NT	≈89	≈103	≈76	≈109
MS19F	*Micromonospora* sp.	-	-	-	-	-	NT	≈121	≈108	≈104	≈80
MS23A	*Streptomyces* sp.	-	-	-	-	-	NT	≈120	≈88	≈104	≈80
MS23B	*Streptomyces* sp.	-	-	-	-	-	NT	≈111	≈104	≈105	≈54

MS23C	*Micromonospora* sp.	-	-	-	-	-	NT	≈121	≈114	≈116	≈99
MS23D	*Micromonospora* sp.	-	-	-	-	-	NT	≈33	≈42	≈51	≈93
MS24	*Micromonospora* sp.	-	-	-	-	-	NT	≈113	≈114	≈104	≈100
MS26H	*Micromonospora* sp.	-	-	-	-	-	NT	≈100	≈104	≈94	≈139
MS26I	*Micromonospora* sp.	-	-	-	-	-	NT	≈86	≈120	≈109	≈140
MS27A	*Streptomyces* sp.	-	-	-	-	+	62.5	≈114	≈69	≈75	≈56
MS29	*Streptomyces* sp.	-	-	-	-	+	15.62	≈84	≈76	≈100	≈108
MS32	*Streptomyces* sp.	-	-	-	-	-	NT	≈74	≈98	≈91	≈121
MS35	*Nocardiopsis sp.*	-	-	-	-	-	NT	≈108	≈112	≈99	≈120
MS38	*Herbiconiux solani*	-	-	-	-	-	NT	≈102	≈89	≈84	≈85
MS39B	*Arthrobacter sp*	-	-	-	-	-	NT	≈125	≈99	≈106	≈74
MS39C	*Arthrobacter sp*	-	-	-	-	-	NT	≈121	≈103	≈107	≈87
MS40A	*Arthrobacter sp*	-	-	-	-	-	NT	≈112	≈92	≈100	≈160
MS40B1	*Arthrobacter sp*	-	-	-	-	-	NT	≈97	≈112	≈95	≈115

MS42	*Micromonospora aurantiaca*	-	-	-	-	-	NT	≈116	≈94	≈114	≈88
MS46	*Micromonospora peucetia*	-	-	-	-	-	NT	≈115	≈109	≈113	≈65
MS48	*Micromonospora* sp.	-	-	-	-	-	NT	≈107	≈100	≈105	≈98
MS49	*Micromonospora* sp.	-	-	-	-	-	NT	≈84	≈49	≈68	≈136
MS50	*Micromonospora* sp.	-	-	-	-	-	NT	≈108	≈74	≈84	≈160
MS51	*Micromonospora* sp.	-	-	-	-	-	NT	≈97	≈51	≈106	≈147
MS52A	*Micromonospora maritima*	-	-	-	-	-	NT	≈63	≈99	≈53	≈98
MS52B	*Micromonospora marina*	-	-	-	-	-	NT	≈67	≈93	≈69	≈152
MS53	*Polymorphospora rubra*	-	-	-	-	-	NT	≈82	≈96	≈101	≈125
MS54	*Streptomyces* sp.	-	-	-	-	+	125	≈99	≈100	≈115	≈71
MS55	*Micromonospora aurantiaca*	-	-	-	-	-	NT	≈83	≈74	≈60	≈103
MS56	*Micromonospora aurantiaca*	-	-	-	-	-	NT	≈69	≈68	≈45	≈12
MS58	*Actinomadura sputi*	-	-	-	-	-	NT	≈84	≈95	≈71	≈125

Legend: -, no inhibition; +, growth inhibition; NT, not tested
[a] Cell viability was assessed using the MTT assay

4. Discussion

The marine environment is a promising source of new microorganisms with potential to produce bioactive compounds. The Portuguese coast is largely unexplored in terms of microbial diversity, and this includes actinobacteria (Prieto-Davó., 2016).

In this study, the actinobacteria associated with marine sediment collected on a beach in Parque Natural do Litoral Norte, in Portugal, was investigated through cultivation-dependent methods. A total of 117 isolates were obtained, of which 52 were identified as actinobacteria. The marine sediment revealed to be rich in terms of actinobacterial diversity, being identified 7 different genera, with the largest fraction of these isolates belonging to the genus *Micromonospora*. The actinobacterial genera recovered from the studied sediment have been reported before in studies with other marine sediments. Prieto-Davó et al. (2016) identified a total of 24 actinobacterial genera in marine sediments collected around the Madeira archipelago, and concluded that the genera *Streptomyces*, *Actinomadura* and *Micromonospora* were the most abundant. Other authors indicate that actinobacteria belonging to the family *Micromonosporaceae* are amongst the most dominant bacteria in marine sediments (Gontang et al., 2007; Jensen et al., 1991; Weyland, 1969). Sediments collected in the North Sea and Chesapeake Bay were found to be rich in *Streptomyces*, *Nocardia*, *Micromonospora* and *Microbispora* species (Weyland, 1969, 1984; Takizawa et al., 1993). The genera *Micromonospora* and *Streptomyces* have been found to be the most dominant in sediments collected from Trondheim Fjord, Norway (Bredholt et al., 2008) and Duncan et al. (2014, 2015) showed that the genera *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Pseudonocardia* and *Streptomyces* were predominant in marine sediments sampled in New Brunswick, Canada.

The three treatment methods used in this study for processing the marine sediment sample were applied in order to inhibit the growth of irrelevant microorganisms and to promote spore germination of actinobacterial strains. These methods have been successfully used by several authors for the isolation of actinobacteria. Xiong et al. (2015) employed 7 different pretreatments, including incubation at 60 ºC for 10 min (method 2 in this study) and incubation with nalidixic acid and cycloheximide at 28 °C for 30 min (method 3 in this study) for the isolation of actinobacteria from marine sediments collected from the Yellow Sea. By using as selective media ABSM4 agar, ISP3 and humic acid-

vitamin agar (HVA), the authors were able to isolate 613 actinobacterial strains of which the genera *Streptomyces*, *Micromonospora, Kocuria*, *Nocardiopsis* and *Saccharomonospora* were found to be the most bioactive ones. Abdelfattah et al. (2016) was able to recover bioactive *Streptomyces* and *Nocardiopsis* species from marine sediments collected from the Red Sea coast by incubating the samples in a water bath at 60 ºC for 10 min (method 2 in this study) and using the selective media HVA and SCN.

The isolation of actinobacteria from various marine sources has been shown to be more efficient when using culture media with low nutrient concentrations, which better simulate the marine environment and avoid the development of fast-growing microorganisms (Gontang et al., 2007; Hames-Kocabas & Uzel, 2012; Jensen et al., 2005). This is the case of the NPS medium used in the present study, which was formulated only with nutrients obtained from the same habitat of the isolated microorganisms. Jensen et al. (2005) used this medium to recover from a marine sediment collected in the island of Guam a high number of salt-dependent actinobacterial isolates belonging to *Micromonospora*, *Streptomyces* and *Actinomadura* genera. Gotang et al. (2007) used an oligotrophic medium, similar to NPS medium, consisting only of sea water and agar to cultivate Gram-positive bacteria from sediments collected in the Republic of Palau from the intertidal zone, 70% (of a total of 199 strains) of the obtained isolates were recovered from this medium, all of them belonging to the genera Actinobacteria and Firmicutes.

The selective media SCN and M3 have been also successfully used for the isolation of actinobacteria, though in this study SCN only allowed the isolation of a small fraction of actinobacterial strains and M3 did not retrieve any isolate. Maldonado et al (2005) employed 6 selective culture media, including M3 and SCN, for the isolation of actinobacteria from marine sediments collected in different locations (Japan Trench, Canary Basin and Norway fjords) (Maldonado el al., 2005), and, contrary to the results of the present study, they were able to obtain a large number of isolates in the M3 and SCN media. Overall, the authors obtained isolates belonging to the genera *Actinomadura*, *Dietzia*, *Gordonia*, *Microbacterium*, *Mycobacterium*, *Nocardiopsis*, *Pseudonocardia*, *Saccharopolyspora*, *Streptosporangium*, *Nonomuraea*, *Verrucosispora* and *Williamsia*.

The treatment methods and the selective media used in this study also allowed the isolation of microbial genera, other than actinobacteria. These microbial genera were found to belong to the phyla Firmicutes (2 genera identified), Proteobacteria (4 genera identified) and Bacteroides (3 genera identified). The phylum Firmicutes includes Gram-positive bacteria, with some members of this group being spore-forming, like the *Bacillus*

and *Paenibacillus* strains recovered in this study, which may explain their resistance to the used treatment methods. The antibiotics used for the isolation of actinobacteria, nalidixic acid and streptomycin, have a broad spectrum against Gram-negative bacteria, but the results obtained indicate that the Proteobacteria and Bacteroidetes isolates recovered in this study are resistant to these antibiotics. As these isolates are not known to produce spores, it is remarkable that some of them could resist to 10 min of incubation at 60 ºC.

Marine actinobacteria are a recognized source of bioactive compounds (Duncan et al., 2015; Hassan & Shaikh, 2017). In this study, all actinobacterial isolates obtained were screened for their antimicrobial and anticancer activities. Antimicrobial assays showed that crude extracts of 6 isolates (strains MS8A1, MS18A, MS18B, MS27A, MS29 and MS54) belonging to the genus *Streptomyces* exhibited activity against *C. albicans*. This is not a surprising result as members of the genus *Streptomyces* are the major producers of bioactive secondary metabolites within the phylum Actinobacteria (Berdy, 2005;2012; Hassan & Shaikh, 2017). A number of secondary metabolites produced by Streptomyces species is derived from strains isolated from marine sediments and, regarding antimicrobial activity, these microorganisms exhibit more frequently activities against fungi and Gram-positive bacteria than against Gram-negative bacteria (Bredholt et al., 2008; Duncan et al., 2015; Duncan et al., 2014; Hassan & Shaikh, 2017; Nakashima et al., 2009; Xiong et al., 2015). Examples of some secondary metabolites produced by marine Streptomyces with antifungal activity against *C. albicans* include isoikarugamycin, a new polycyclic tetramic acid macrolactam that is effective at a MIC value of 2-4 μg mL^{-1} (Lacret et al., 2014), daryamides A and B, exhibiting MIC values of 62.5 and 125 μg mL^{-1}, respectively (Asolkar et al., 2006; Mitchell et al., 2004), 4-phenyl-1-napthyl-phenyl acetamide, which has a strong antifungal activity particularly against *C. albicans* (Dhanasekaran et al., 2008). The comparison of the MIC values of the cited compounds with the ones obtained in this study (values in the range of 125-3.90 μg mL^{-1}), suggest that the crude extracts of the isolated *Streptomyces* strains may have a high bioactivity potential, especially given the fact that these MIC values were tested with crude extracts and not with pure compounds, as is the case of the compounds indicated above. In the future, it will be important to investigate if new secondary metabolites may be present in these extracts.

Screening of anticancer activity showed that crude extracts (tested at a concentration of 15 μg mL^{-1}) of strains MS3B, MS3E and MS56 belonging to the genus *Micromonospora*, and MS18B, identified as a *Streptomyces* sp., had promising anticancer activities. After an

incubation period of 48 h, crude extracts of strains MS3B, MS3E and MS56 led to a cell viability of 8% and 5% in the cell line T47-D, and 12% in the cell line SH-SY5Y, respectively, while strain MS18B induced cell viabilities of, respectively, 8% and 2% in the two cancer cell lines tested. *Micromonospora* species isolated from marine sediments are known to produce diverse anticancer compounds, such as diazepinomycin, an alkaloid compound with high antitumor activity, lagumycin B, an angucycline antibiotic with cytotoxicity against diverse cancerous and non-cancerous cell lines and streptonigrin, a natural compound with potent activity against human neuroblastoma SH-SY5Y cell line (McAlpine et al., 2008; Mullowney et al., 2015; Wang et al., 2002) A vast number of secondary metabolites with anticancer activity has been described for *Streptomyces* strains isolated from marine sediments, such as daryamides, with moderate cytotoxicity against human colon carcinoma cell line, and aureoverticillactam and chromomycin B, which exhibit high cytotoxic activity against several tumor cell lines (Asolkar et al., 2006; Lu et al., 2012; Mitchell et al., 2004).

As with the crude extracts that showed antimicrobial activity, in the future it will be important to better assess the anticancer activity of the most promising extracts and to investigate if new anticancer compounds may be responsible for these activities. To the best of our knowledge this is the first study on the isolation of actinobacteria from marine sediment of the Portuguese northern coast and investigation of their bioactive potential, namely antimicrobial and anticancer.

5. Conclusion

This study focused on the isolation of actinobacteria from marine sediment of the Portuguese northern coast and on the screening of their potential to produce bioactive compounds. In total, 117 isolates were recovered, of which 52 strains were identified as actinobacteria. The marine sediment showed to have a high diversity of actinobacteria, having been identified 7 different genera, with the majority of the isolates belonging to the genus *Micromonospora*. Results revealed that the three treatment methods and culture media used in this study allowed the recovery of isolates belonging to the genera *Micromonospora*, *Streptomyces*, *Arthrobacter*, *Polymorphospora*, *Herbiconiux*, Nocardiopsis and *Actinomadura*. Most actinobacterial isolates were recovered from NPS medium, indicating that isolation of these microorganisms is more effective when using

nutrient-poor culture media that better mimics the marine environment. Only a small fraction of actinobacterial isolates could be recovered from SCN medium, while no growth was obtained in M3 medium. Besides actinobacterial isolates, the treatment methods and selective culture media used in this study also allowed the isolation of bacterial genera belonging to the phyla Firmicutes, Proteobacteria and Bacteroides.

Crude extracts of strains MS8A1, MS18A, MS18B, MS27A, MS29 and MS54 belonging to the *Streptomyces* genus showed activity against *C. albicans*. MIC values obtained (in the range of 125 - 3.90 μg mL^{-1}) show that crude extracts resultant from these *Streptomyces* isolates may have a high bioactive potential, being now necessary to investigate the presence of new bioactive molecules in these extracts. These results confirm the remarkable capacity of Streptomyces species to produce bioactive compounds.

Screening of anticancer activity showed that crude extracts of strains MS3B, MS3E and MS56 belonging to the genus *Micromonospora*, and MS18B, identified as *Streptomyces* sp., had promising anticancer activities, especially strain MS18B which presented cell viability values lower than 10% in the two cell lines tested. In the future, it will be important to better investigate the antimicrobial and anticancer activities of the crude extracts of the most promising strains and the possible presence of new compounds.

The results obtained in this study will contribute to increase the scant information available on culturable actinobacteria associated with marine sediments of the Portuguese coast and of their bioactive potential.

CHAPTER 3

ISOLATION AND INDENTIFICATION OF RARE ACTINOBACTERIA FROM THE MARINE SPONGE *H.PERLEVIS*

1. Introduction

The excessive and undue use of antibiotics is leading to an increase in multidrug-resistant bacterial infections, for which new therapeutic alternatives are clearly needed. In addition, there is an enormous need to find effective and less aggressive treatments against many serious diseases, such as cancer (Duncan et al., 2015). These problems demand an urgent search for new treatment strategies and the discovery of new natural compounds with relevant pharmacological activities is an important route to achieve this goal (Abdelmohsen et al., 2010b; Undabarrena et al., 2016).

The phylum Actinobacteria, and especially the class Actinobacteria, is well known for its great capacity to produce secondary metabolites with a wide range of bioactivities, many of these compounds having a significant role in human health. Among these microorganisms, members of the genus *Streptomyces* are considered the major producers of relevant bioactive compounds, being responsible for the production of more than 10 400 bioactive metabolites (Berdy, 2012). Due to their outstanding biosynthetic potential, *Streptomyces* species have been extensively explored in the last decades, which has resulted in a marked decrease in the discovery of new compounds from these microorganisms (Berdy, 2005). In order to increase the discovery rate of new bioactive compounds, it is important to focus the research in other actinobacterial genera, mainly in rare genera of actinobacteria, since the biosynthetic potential of these microorganisms is underexplored (Subramani & Aalbersberg, 2013).

The term rare actinobacteria usually refers to non-*Streptomyces* species or to actinobacterial strains that are isolated less frequently using conventional culture-dependent methods (Berdy, 2005; Dhakal et al., 2017). In the recent decades, through the employment of isolation approaches that favor the enrichment of rare actinobacteria, a high diversity of these microorganisms has been reported in the literature. These microorganisms are responsible for the production of more than 3000 different bioactive metabolites (Berdy, 2005;2012; Tiwari & Gupta, 2013). Examples include pseudonocardians, dermacozines, marinacarbolines, neomaclafungin, etc., produced by microorganisms belonging to the genera *Pseudonocardia, Dermacoccus Marinactino*spo*ra* and *Actinoalloteichus* and exhibiting various bioactivities such as antibacterial, cytotoxic, antioxidant, antimalarial and antifungal, being (Abdel-Mageed et al., 2010; Huang et al., 2011; Li et al., 2011; Sato et al., 2012).

Unexplored or underexplored habitats, such as marine ecosystems, are considered major sources of rare actinobacteria and of new bioactive compounds with biotechnological importance (Dhakal et al., 2017).

Marine sponges belong to the phylum Porifera are one of the oldest multicellular living beings in the world. These sessile and filtering organisms are known to develop various symbiosis relationships with bacteria, and these associations are so important that microbial abundance in sponges may account up to half of their weight (Abdelmohsen et al., 2010a). Marine sponges are a known source of biologically active metabolites, but in some cases the production is carried out by their microbial symbionts rather than by the sponge itself (Wilson et al., 2014). These organisms have been described to host a wide variety of actinobacteria, many of them being rare and new (Abdelmohsen et al., 2014). Examples of rare actinobacteria isolated from marine sponges include species of *Nocardiopsis*, *Actinomadura*, *Salini*spo*ra* and *Verrucosi*spo*ra*, and many of them were shown to be capable of producing secondary metabolites with antimicrobial and cytotoxic activities, like nocapyrones, bendigoles, rifamycins and thiocoraline (Kim et al., 2006; Schneemann et al., 2010; Simmons et al., 2011; Wyche et al., 2011).

This study had as objective the isolation and identification of rare actinobacteria associated with the marine sponge *Hymeniacidon perlevis*, collected in the intertidal zone of a beach in northern Portugal. This species of sponge is distributed along the Atlantic Ocean, Mediterranean coast and the Yellow Sea, and usually colonizes rocks or other hard surfaces buried in marine sediments (Alex et al., 2013). Previous studies have shown a large diversity of actinobacteria in samples of *H. perlevis* obtained from the Yellow Sea South China Sea and Atlantic Ocean (Alex et al., 2013; Sun et al., 2010; Xi et al., 2012; Xin et al., 2008; Xin et al., 2009; Zhang et al., 2006), however, to the best of our knowledge, rare actinobacteria associated with this sponge inhabiting the Portuguese coast has never been investigated before.

2. Materials and Methods

2.1. Sample collection

The sponge *H. perlevis* was collected in the intertidal zone of Memória beach, in Matosinhos, Portugal (41°13.840157'N, 8°43.331863'W). The sponge was transferred to a

sterile plastic bag, transported to the laboratory in a cooling box and processed within an interval of two hours.

The sponge was identified by an experienced researcher as belonging to the species *Hymeniacidon perlevis*, through the analysis of its morphological characteristics.

2.2. Isolation of rare actinobacteria

The sponge was initially washed, under sterile conditions, with sterile marine water to remove looser particles. Three fragments with ca. 1.5 g each were then cut, weighted and homogenized separately in a sterile mortar with 2 mL of sterile seawater. Each macerated fragment was then subjected to one of the following pretreatments: incubation in a microwave (2450 MHz) for 3 min at 120 W (pretreatment 1); incubation in a water bath at 60 ºC for 30 min (pretreatment 2) and incubation in an oven at 120 °C for 60 minutes (pretreatment 3). The resulting samples were then ten-fold diluted to 10^{-4} and an aliquot of 100 µl of each dilution was spread over the surface of the following selective isolation media: M1 agar (per liter of seawater): 10 g of soluble starch, 4 g of yeast extract, 2 g of peptone and 17 g of agar; M2 agar (per liter, with a ratio of seawater:deionized water of 90:10): 6 mL of glycerol, 1 g of arginine, 1 g of K_2HPO_4, 0.5 g of $MgSO_4$ and 18 g of agar; Starch-casein-nitrate agar (SNC) (per liter, with a ratio of seawater:deionized water of 60:40): 10 g of soluble starch, 0.3 g of casein, 2 g of K_2HPO_4, 2 g of KNO_3, 2 g of NaCl, 0.05 g of $MgSO_4.7H_2O$, 0.02 g of $CaCO_3$, 0.01 g of $FeSO_4.7H_20$ and 17 g of agar. All media were supplemented with cycloheximide (50 mgL^{-1}), acid nalidixic (50 mgL^{-1}) and nystatin (50 mgL^{-1}) in order to inhibit the growth of fungi and Gram-negative bacteria. The plates were incubated at 28 °C for a period of 4 months.

Along the incubation period, plates were periodically inspected by visual observation and all colonies with different morphological characteristics were picked and re-streaked numerous times in the same agar medium until obtainment of pure colonies. Each isolate was cryopreserved at -80 ºC in 30% glycerol. Before the cryopreservation step each isolate was grown in 5 mL of liquid medium, M1, M2 or SCN, according to the respective isolation medium, at 25 ºC, 100 rpm, for one week and 1 mL of grown culture was used for cryopreservation.

2.3. Identification of isolated strains by 16S rRNA gene sequencing

Genomic DNA was extracted from all isolated microorganisms using the E.Z.N.A.® Bacterial DNA Kit, (Omega Biotek, Norcross, GA*)*, following the manufacturer's instructions, with a few modification steps: (i) before starting the extraction protocol, samples were incubated at 95 °C for 10 min, followed by incubation on ice for more 10 min; (ii) in the step of lysozyme addition, the samples were incubated at 37 °C for 30 min, instead of 10 min as mentioned in the kit protocol; (iii) in the optional step used for bacteria difficult to lyse, two Zirconia beads (2.3 mm diameter) were added along with the glass beads and the samples were vortexed for 10 min; (iv) incubation with proteinase K was made with a concentrated stock (10 mg mL^{-1}) instead of the solution provided in the kit and was extended up to 2h (v) the centrifugation speeds described in the kit protocol were increased in all steps from 10,000 g to 13,000 g; (vi) in the final step of DNA elution, 25 µL of elution buffer were added to the HiBind® DNA Mini Column, instead of 50-100 µL as indicated in the protocol (step performed twice).

Biomass for DNA extraction was obtained through centrifugation of 2 ml of the liquid cultures (7.000 g for 5 min) previously grown for cryopreservation purposes. 16S rRNA gene was amplified by PCR using the universal primers 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane, 1991). The PCR mixture (total volume of 10 µL) contained: 5 µL of Taq PCR Master Mix (Qiagen, Valencia, CA), 1 µL of primer 27F (2 µM), 1 µL of primer 1492R (2 µM) and 3 µL of DNA template. Negative controls were also conducted and consisted in replacing in the PCR mixture the DNA template by 3 µL of Nuclease-Free Water. PCR conditions were as following: initial denaturation at 95 °C for 15 min, followed by 30 cycles at 94 °C for 30 s, 48 °C for 90 s, 72 °C for 90 s and a final extension at 72 °C for 10 min. PCR was performed in an Applied Biosystems ThermoCycler (Applied Biosystems Inc., Foster City, CA, USA). PCR products were separated on a 1.5% agarose gel containing SYBR Safe (ThermoFisher Scientific, USA), at 150 V for 30 min. Purification and sequencing of the amplified DNA was performed by GenCore, I3S (Instituto de Investigação e Inovação em Saúde, Portugal). The obtained 16S rRNA sequences were analyzed using the Geneious software, version 4.8.2 and the resulting consensus sequences were compared to the GenBank database using the blastn algorithm. The consensus sequences were additionally compared with the databases EzTaxon (http://www.ezbiocloud.net/) and

Ribosomal Database Project (https://rdp.cme.msu.edu/index.jsp) in order to confirm the results obtained in the GenBank database.

All the sequences and their five closest neighbor sequences in Genbank were aligned using the Geneious software. The alignment was used to generate a Maximum Likelihood phylogenetic tree of 1366bp, performed as the defaults of the ATCG South of France bioinformatics platform (http://www.atgc-montpellier.fr/) with 100 bootstraps.

3. Results

The isolation of rare actinobacteria from the marine sponge, *H. perlevis*, was performed using three different pretreatments and three selective culture media. Along an incubation period of 4 months, a total of 188 isolates with distinct morphological characteristics were obtained. Most of the colonies grown in the culture media exhibited morphological characteristics typical of actinobacteria, such as presence of spores or hyphae, colonies with a leather texture and with diverse colors and alteration of the color of the media. Figure 11, shows examples of some of the colonies isolated.

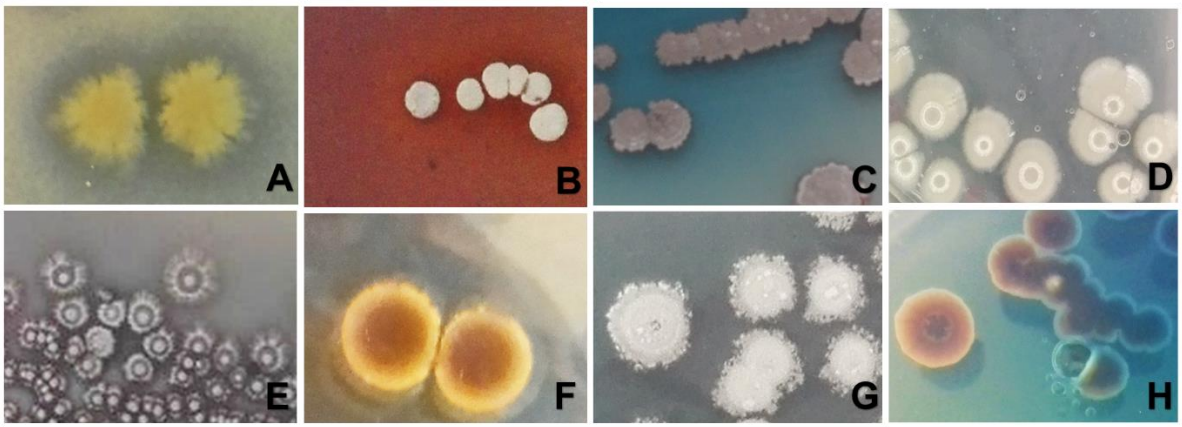

Figure 11 - Examples of some isolates obtained from *H. perlevis.* (A) Strain E101, (B) strain E172, (C) strain E230, (D) strain E69, (E) strain E102, (F) strain E207, (G) strain E209 and (H) strain E221.

The distribution of the number of recovered isolates according to the pretreatments and culture media employed is presented in Fig. 12. By analysing this figure it is possible to observe that the pretreatment that allowed a higher recovery of isolates was pretreatment 1 (microwave incubation), with 125 isolates being obtained. Pretreatment 2 (incubation at

60 ºC) led to the isolation of 61 different colonies, while incubation at 120 ºC was shown to be highly selective, only allowing the recovery of 2 isolates. The selective media employed led to a more or less even distribution of the recovered isolates, though medium M1 always had a sligthly higher number of isolates.

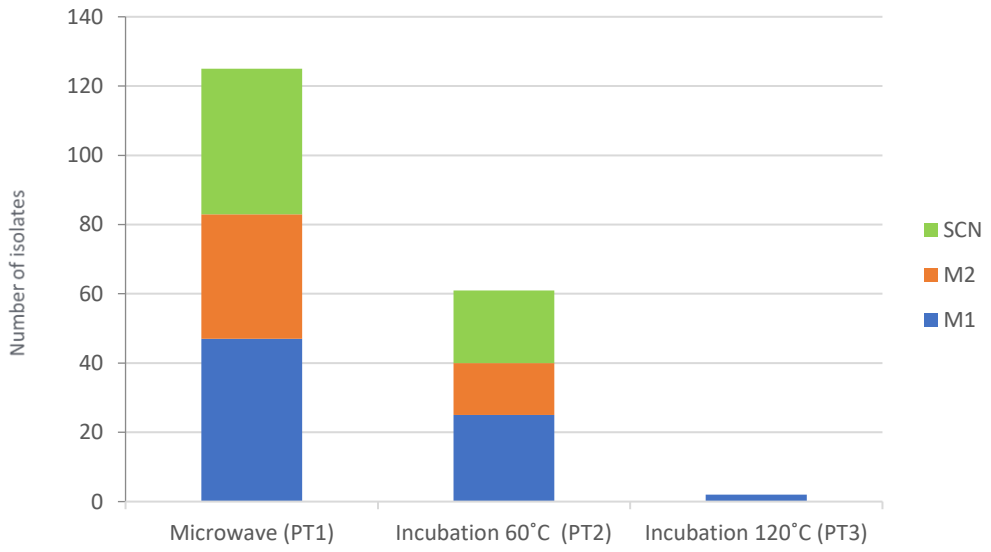

Figure 12 - Number of isolates recovered according to the pretreatments and culture media used in this study for the isolation of rare actinobacteria from the marine sponge *H. perlevis*.

For taxonomical identification of the obtained isolates the nearly full-length (>1300 bp) 16S rRNA gene was sequenced. To date, it has only been possible to identify 18 isolates, of which 17 were found to belong to the phylum Actinobacteria and only one was identified as belonging to the phylum Firmicutes (Table 4 and Fig 13). The identification results of these isolates also revealed that the 17 actinobacterial strains identified so far have a high phylogenetic diverstiy, belonging to 5 different families (*Streptomycetaceae*, *Nocardiopsaceae*, *Tsukamurellaceae*, *Brevibacteriaceae* and *Nocardiopsaceae*) and 6 genera (*Streptomyces*, *Nocardia, Gordonia*, *Tsukamurella*, *Brevibacterium* and *Nocardiopsis*). Interestingly , with the exception of the genus *Streptomyces*, all the other genera constitute rare actinobacterial genera.

Table 4 - Taxonomic identification of some isolates obtained from the marine sponge *H. perlevis*

Isolate	Pretreatment	Culture medium	Phylum	Identification	Similarity %
E67	Microwave	M2	Actinobacteria	*Nocardia* sp.	99.0
E74	Microwave	M2	Actinobacteria	*Streptomyces* sp.	99.8
E79	Microwave	M2	Actinobacteria	*Nocardiopsis prasina*	99.8
E82	Microwave	M2	Actinobacteria	*Streptomyces* sp.	100
E85	Microwave	M2	Actinobacteria	*Streptomyces* sp.	99.8
E87	Microwave	M2	Actinobacteria	*Brevibacterium* sp.	98.9
E194	Microwave	M2	Actinobacteria	*Nocardia* sp.	99.0
E229	Microwave	M2	Actinobacteria	*Streptomyces thermocarboxydus*	99.8
E247	Microwave	M2	Actinobacteria	*Streptomyces* sp.	100
E99	Microwave	SCN	Actinobacteria	*Streptomyces* sp.	100
E107	Microwave	SCN	Actinobacteria	*Streptomyces* sp,	100
E109	Microwave	SCN	Actinobacteria	*Gordonia* sp.	99.0
E117	Microwave	SCN	Actinobacteria	*Tsukamurella* sp.	99.3
E119	Microwave	SCN	Actinobacteria	*Streptomyces* sp	99.9
E122	Microwave	SCN	Actinobacteria	*Streptomyces niveus*	99.6
E171	Incubation 60ºC	SCN	Actinobacteria	*Streptomyces* sp.	100
E199	Incubation 60ºC	SCN	Firmicutes	*Bacillus licheniformis*	99.1
E202	Incubation 60ºC	SCN	Actinobacteria	*Streptomyces* sp.	100

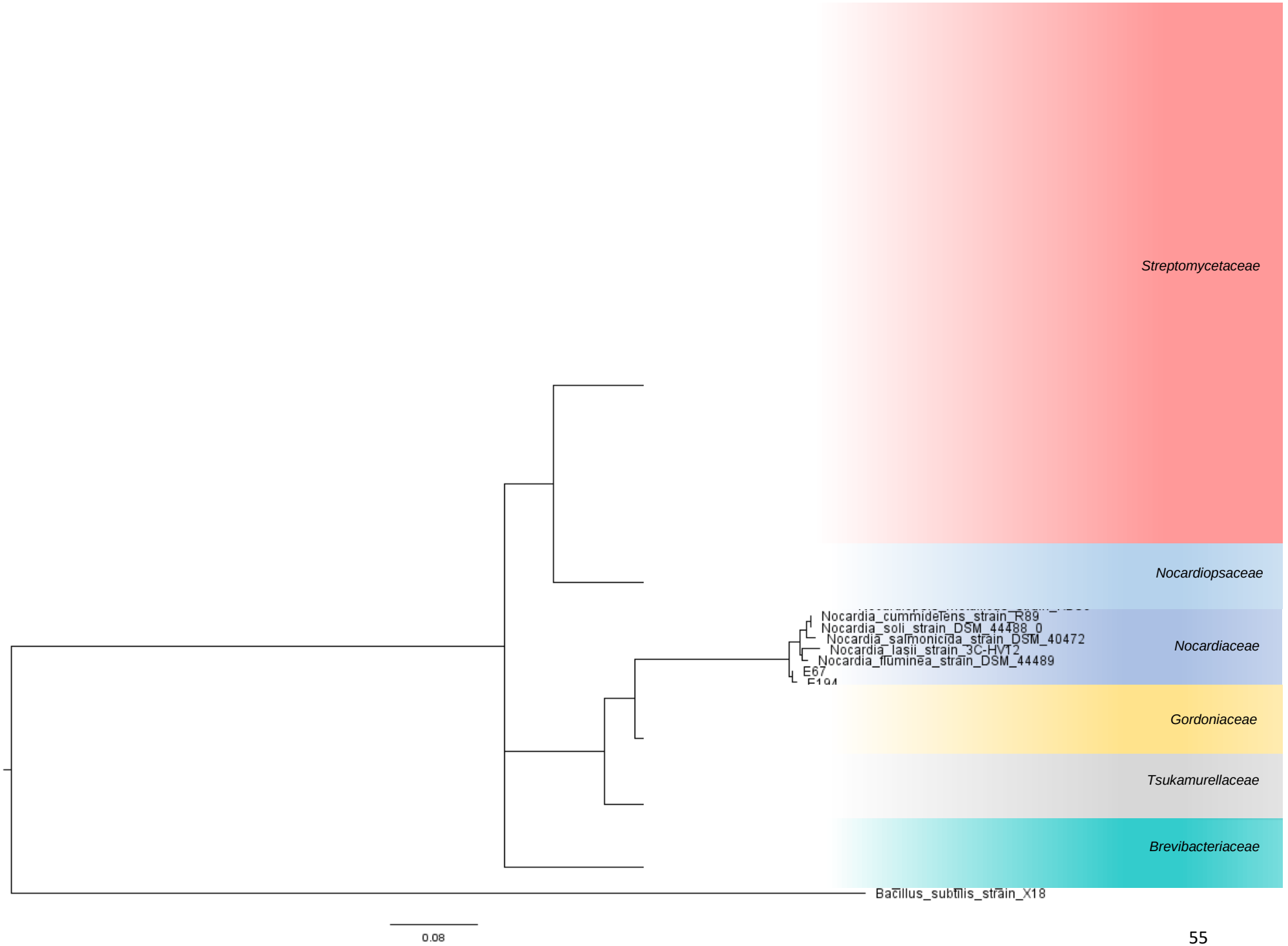

Figure 13 - **Phylogenetic tree of identified actionobacterial strains isolated from sponge *H. perlevis* and their GenBank nearest neighbor**. Maximum Likelihood phylogenetic tree was performed using 79 sequences with 1366bp and 100 bootstraps. *Bacillus subtilis* was used as an out group.

4. Discussion

Different pretreatments and selective culture media are usually employed for the isolation of rare actinobacteria (Subramani & Aalbersberg, 2013). In this study, the pretreatments used for the isolation of rare actinobacteria from the marine sponge, *H. perlevis*, showed to have a high influence in the number of retrieved isolates, with microwave incubation allowing the isolation of the largest number of different colonies. Several studies have demonstrated that microwave irradiation promotes the isolation of rare actinobacteria, such as *Micromono*spo*ra, Micropoly*spo*ra, Nocardia*, *Actinomadura*, *Strepto*spo*rangium* and *Lentzea* sp. (Bulina et al. 1997; Yang et al. 2008; Xue et al. 2010; Wang et al. 2013). Nonethless, some of these studies have also showed that this pretreatment could increase as well the isolation of *Streptomyces* sp. In the present study, microwave irradiation (pretreatment 1) led to the isolation of *Nocardia*, *Gordonia, Tsukamurella*, *Nocardiopsis* and *Brevibacterium* sp.*,* which are considered rare actinobacterial genera, but also *Streptomyces* sp., which is in accordance with previous studies.

The application of pretreatments consisting in the heating of samples are described in the literature to be selective for the isolation of sporulating bacteria, having also shown efficient for the isolation of rare actinobacteria (Jiang et al., 2016). Imada et al. (2010) incubated samples of marine sediments at 55 ºC for 30 min and was able to isolate microorganisms belonging to the genera *Micromonospora* and *Streptomyces.* Jiang et al. (2016) reported that the incubation of samples of water or soil suspensions at 60 ºC for 30 min was effective in the isolation of *Micromonospora* sp. Eccleston et al. (2008) was able to select several genera of the family *Micromonosporae* by combining different pretreatments, one of which consisting in the incubation of samples of aquatic sediments, at 55 ºC for periods of 30 or 60 min. In this study, incubation of a sample of *H. perlevis* at 60 ºC for 30 min resulted, until the moment, in the isolation of bacterial strains belonging to the genus *Streptomyces*, which is not a surprising result due to the fact that these microorganisms produce spores that may withstand this temperature. However, as only 3 strains retrieved from this pre-treatment have been identified so far, it is not possible to conclude on the efficacy of this treatment on the isolation of rare actinobacteria.

Incubation of a sample of *H. perlevis* at 120 ºC for 60 min (pretreatment 3) was shown to be extremely selective in terms of recovery of different isolates. This treatment was applied in this study with the objective of eliminating spores of *Streptomyces* species as these

microorganisms usually have a faster growth when compared with rare actinobacteria genera, and may thus prevent their growth (Hayakawa et al., 1991; Nonomura, 1969). This pre-treatment has been demonstrated before to be a good approach for the isolation of rare actinobacteria, such as *Strepto*sp*orangium* sp., once the sporangiospores that these species produce are capable of withstanding and resisting harsh physical and chemical pretreatments (Hayakawa et al., 1991). Similarly, conidia producing species, such as *Dactylo*sp*orangium* sp. have also shown to have a high survival rate when exposed to this pre-treatment, with 140 species of this genus having been isolated by applying this pretreatment (Hayakawa et al., 1991). In another study, Niyomvong et al. (2012) showed that combining different pretreatments, such as incubation in a water bath at 50 °C for 6 min, followed by dry incubation at 120 °C for 1 h and addition of 1.5% phenol, resulted in a lower recovery of non-target bacteria and increased the isolation of rare actinobacteria belonging to the genera *Actinoplanes*, *Gordonia*, *Microbio*spo*ra*, *Micromono*spo*ra*, *Nocardia* and *Nonomuraea*. Though the isolates retrieved from pre-treatment 3 have not yet been identified, according to the literature, there are good chances that these isolates belong to rare actinobacteria genera, and they may be very promising for the production of bioactive compounds.

Regarding the extraction of DNA from the various isolates, the use of an optimized protocol proved to be more efficient, as it was possible to obtain a higher quantity of extracted DNA. Sequencing of the 16S rRNA gene is widely used in phylogenetic studies since this region of DNA is characteristically well conserved in different bacterial species, having at the same time several zones with specific polymorphisms that allow an adequate evaluation of the phylogenetic relationships between bacteria. Phylogenetic analyses of the isolates so far identified revealed that 11 isolates are *Streptomyces* sp., having been obtained from pretreatments 1 and 2. Although not considered rare actinobacteria, this genus is the largest producer of bioactive compounds belonging to the phylum Actinobacteria, so it will always be important to test in the future the antimicrobial and anticancer activities associated with these isolates (Dhakal et al., 2017; Hassan & Shaikh, 2017). Nonetheless, the fact that these microorganisms have been intensively explored reduces the probability of finding new bioactive compounds. Taxonomic results also showed that some isolates obtained from the sponge *H. perlevis*, belonged to rare actinobacteria genera, namely to the genera *Nocardia, Gordonia, Tsukamurella*, *Brevibacterium* and *Nocardiopsis*, indicating that the experimental approach used in this study seems to be suitable for the selection of rare actinobacteria.

The actinobacterial community associated with the sponge *H. perlevis* inhabiting the Yellow sea and the South China Sea has been studied before by some authors (Sun et al. (2010), Xi et al. (2012), Xin et al. (2009) and Zang et al. (2006). Similarly, to the present study, the authors used different selective culture media but no pretreatments were applied, except in the study of Xi et al. (2012), where the samples were incubated in a water bath at 55 °C for 6 min. In these studies, several actinobacterial genera associated with *H. perlevis* were isolated, namely *Streptomyces, Nocardiopsis*, *Gordonia*, *Nocardia*, *Micromonos*p*ora*, *Pseudonocardia*, *Salinispora*, *Mycobacterium* and *Rhodococcus*, with the first 4 genera being also identified in the present study. Sun et al. (2010) used in their study seven different selective culture media, including the M1 and M2 media also used in this study. In contrast to our results, the authors reported that the medium allowing the greatest diversity and largest number of actinobacteria was M2, while the medium M1 did not led the isolation of colonies with specific characteristics of actinobacteria.In the study conducted by Zhang et al. (2006), eight selective culture media were used, also including the M1 and M2 media. Medium M1 led to the isolation of microorganisms belonging to 4 different genera: *Streptomyces*, *Nocardiopsis*, *Nocardia* and *Micromonos*p*ora*, while M2 retrieved isolates belonging to 7 actinobacterial genera: *Rhodococcus*, *Micromonos*p*ora*, *Nocardia*, *Pseudonocardia*, *Nocardiopsis* and *Streptomyces*.

In order to conclude the present study, the remaining isolates will be identified and the bioactive potential, namely antimicrobial and anticancer activities, of all isolates identified as actinobacterial species will be investigated.

5. Conclusion

This study focused on the isolation and identification of rare actinobacteria from the marine sponge *H. perlevis*. Of the 188 strains isolated from this sponge, only 18 were possible to be taxonomically identified, revealing the isolation of rare genera of actinobacteria, such as *Nocardia*, *Nocardiopsis*, *Brevibacterium*, *Gordonia* and *Tsukamurella*, which are quite promising regarding the production of bioactive compounds. Results revealed that the three pretreatments applied for maximizing the chances of isolating rare actinobacteria had a high influence in the number of recovered isolates and that microwave incubation was the pretreatment that led to the largest diversity of microbial colonies. This pre-treatment allowed the isolation of the actinobacterial genera previously indicated, and also *Streptomyces* sp. Though this last genus is not considered rare, due to its prolific capacity

to produce bioactive compounds, it will be always interesting to investigate the bioactive potential of the *Streptomyces* strains obtained in this study. Incubation of *H. perlevis* at 60 ºC resulted, so far, in the isolation of *Streptomyces* sp., however the majority of the microbial strains retrieved from this pre-treatment has not been identified yet, and so the results obtained do not allow drawing consistent conclusions on the efficacy of this pre-treatment to isolate rare actinobacteria. Incubation of *H. perlevis* at 120 ºC has shown to be very selective, only leading to the isolation of two different colonies but, according to the literature, there are high chances of these isolates being rare actinobacteria.

As future work, it will be necessary to identify the remaining isolates and to test their ability to produce compounds with antimicrobial and anticancer activity.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

1. General discussion

Natural compounds are the most important resources for the discovery of new drugs, such as antibiotics to combat resistant bacteria and anticancer agents to combat cancer. A large fraction of the drugs commercially available in the last decades has a natural origin or is inspired in natural molecules. Bacteria are at the top of the organisms producing natural compounds, with the phylum Actinobacteria being the most renowned one in the production of secondary metabolites with various important bioactivities at the pharmaceutical and industrial level. The genus Streptomyces is the most known due to its wide capacity to produce bioactive compounds with different bioactivities, however rare actinobacteria have been also demonstrated to be important contributors for the production of bioactive compounds.

Due to the overexploitation of terrestrial environments, a great interest has arisen in recent years in the prospection of microorganisms from marine environments and, in this context, marine actinobacteria have already shown to be an important source of bioactive compounds. In this thesis, two different marine samples, coastal marine sediment and a marine sponge, both collected in the coastal area of northern Portugal, were explored in terms of cultivable actinobacteria in order to assess their potential to produce bioactive compounds with antimicrobial and anticancer activity. The Portuguese coast is largely unexplored regarding microbial diversity, including actinobacteria, and their bioactive potential. The results of the first study conducted in this thesis revealed that the marine sediment collected on a beach on Parque Natural do Litoral Norte presented a high diversity of actinobacteria and that some of these isolates had a promising antimicrobial and anticancer potential, as shown by the results obtained with crude extracts of some isolates belonging to the genera Streptomyces and Micromonospora. These results are very interesting, being now important to analyze these crude extracts in order to investigate the potential presence of new bioactive substances. In addition, the results obtained in this study indicate that more efforts should be undertaken to bioprospect other marine sediments from the Portuguese coast. The second study presented in this thesis focused on the isolation and identification of rare actinobacteria from the marine sponge *H. perlevis*. The results showed that the different pretreatments and selective culture media employed to maximize the chances of recovering rare actinobacteria allowed the isolation of a large number of strains. These isolation techniques seem to be promising for the selection of rare actinobacteria, since the taxonomic identification of some isolates has

revealed the recovery of several actinobacteria genera considered rare. Though the results obtained are premature, these suggest that the marine sponge *H. perlevis* may be rich in actinobacteria.

2. Conclusion

This thesis focuses on the isolation of actinobacteria from different sources of the Portuguese northern coast and on the study of their bioactive potential which, to our knowledge, has never been investigated before.

The marine sediment collected from Parque Natural do Litoral Norte proved to have a high diversity of actinobacteria, with some of them showing a promising bioactive potential, and the results obtained point to the importance of bioprospecting other coastal marine sediments in Portugal. The high ability of actinobacteria to produce bioactive secondary metabolites, reported by numerous authors, was also observed in this study.

The different pretreatments and selective culture media used to isolate rare actinobacteria from the sponge *H. perlevis* allowed the isolation of some rare actinobacterial genera, though the vast majority of the isolates has not yet been identified. The results so far obtained also suggest that the studied sponge seems to be rich in actinobacteria.

As future work, it will be necessary to deeply investigate the bioactive potential of the most promising crude extracts and to analyze the presence of new bioactive substances. It will be also important to identify the remaining isolates recovered from the marine sponge *H. perlevis* and to screen the bioactive potential of their crude extracts. Overall, the results obtained in this thesis contribute to the increase of the scarce information available on culturable actinobacteria associated with marine sediments and sponges of northern Portuguese coast as well as on the bioactive potential of some of these isolates.

CHAPTER 5

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